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(54) Title: RETROVIRAL GENE THERAPY VECTORS AND THERAPEUTIC METHODS BASED THEREON																	
<table border="1" style="margin: auto;"><tr><td style="text-align: center;">LTR</td><td style="text-align: center;"> </td><td style="text-align: center;"> </td><td style="text-align: center;">GAG</td><td style="text-align: center;">POL</td><td style="text-align: center;">ENV</td><td style="text-align: center;">LTR</td></tr><tr><td colspan="2" style="text-align: center;">5'ss</td><td colspan="2" style="text-align: center;">AUGgag</td><td colspan="3" style="text-align: center;">3'ss'</td></tr></table>				LTR			GAG	POL	ENV	LTR	5'ss		AUGgag		3'ss'		
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(57) Abstract <p>Retroviral vectors are disclosed which include an insertion site for genes of interest and are capable of expressing high levels of the protein derived from the genes of interest in a wide variety of transfected cell types. Also disclosed are retroviral vectors lacking a selectable marker, thus rendering them suitable for human gene therapy in the treatment of a variety of disease states without the co-expression of a marker product, such as an antibiotic. These retroviral vectors are especially suited for use in certain packaging cell lines.</p>																	

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**RETROVIRAL GENE THERAPY VECTORS AND THERAPEUTIC
METHODS BASED THEREON**

CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application is a continuation-in-part of co-owned and co-pending United States application 07/786,015, and filed October 31, 1991, which is a continuation-in-part of co-owned and co-pending United States application 07/607,252, filed October 31, 1990,
10 and which is a continuation-in-part of co-owned United States application 07/131/926, filed December 11, 1987, now abandoned, each of which applications are hereby incorporated by reference herein.

15 **1. INTRODUCTION**

 The present invention is directed to gene therapy vectors and methods, and provides a family of novel recombinant retroviral vectors capable of efficiently transferring any gene of interest into a wide range of
20 mammalian target cells. Cells transduced with the recombinant retroviral vectors of the invention are capable of expressing high levels of a desired gene product for long periods of time. Thus, such transduced cells may be useful in the treatment of a
25 wide variety of diseases wherein permanently augmenting or adding the production of a given protein or other polypeptide is therapeutically desirable. Preferred vectors of the invention lacking selectable markers are described, and are particularly useful for
30 somatic cell gene therapy in the treatment of diseases wherein the co-production of marker gene products, such as antibiotics, would be undesirable or unacceptable.

35

2. BACKGROUND OF THE INVENTION

Numerous methods exist for genetically engineering mammalian cells. There is great interest in genetically engineering mammalian cells for several reasons including the need to produce large quantities of various polypeptides and the need to correct various genetic defects in the cells. The methods differed dramatically from one another with respect to such factors as efficiency, level of expression of foreign genes, and the efficiency of the entire genetic engineering process.

One method of genetically engineering mammalian cells that has proven to be particularly useful is by means of retroviral vectors. Retrovirus vectors and their uses are described in many publications including Mann, et al., Cell 33:153-159 (1983) and Cone and Mulligan, Proc. Natl. Acad. Sci. USA 81:6349-6353 (1984). Retroviral vectors are produced by genetically manipulating retroviruses.

Retroviruses are RNA viruses; that is, the viral genome is RNA. This genomic RNA is, however, reverse transcribed into a DNA copy which is integrated stably and efficiently into the chromosomal DNA of transduced cells. This stably integrated DNA copy is referred to as a provirus and is inherited by daughter cells as any other gene. As shown in Figure 1, the wild type retroviral genome and the proviral DNA have three Psi genes: the gag, the pol and the env genes, which are flanked by two long terminal repeat (LTR) sequences. The gag gene encodes the internal structural (nucleocapsid) proteins; the pol gene encodes the RNA directed DNA polymerase (reverse transcriptase); and the env gene encodes viral envelope glycoproteins. The 5' and 3' LTRs serve to promote transcription and polyadenylation of virion RNAs.

- Adjacent to the 5' LTR are sequences necessary for reverse transcription of the genome (the tRNA primer binding site) and for efficient encapsidation of viral RNA into particles (the Psi site). Mulligan, R.C., In: Experimental Manipulation of Gene Expression, M. Inouye (ed), 155-173 (1983); Mann, R., et al., Cell, 33:153-159 (1983); Cone, R.D. and R.C. Mulligan, Proceedings of the National Academy of Sciences, U.S.A., 81:6349-6353 (1984).
- 10 If the sequences necessary for encapsidation (or packaging of retroviral RNA into infectious virions) are missing from the viral genome, the result is a cis acting defect which prevents encapsidation of genomic RNA. However, the resulting mutant is still capable
- 15 of directing the synthesis of all virion proteins. Mulligan and coworkers have described retroviral genomes from which these Psi sequences have been deleted, as well as cell lines containing the mutant genome stably integrated into the chromosome.
- 20 Mulligan, R.C., In Experimental Manipulation of Gene Expression, M. Inouye (ed), 155-173 (1983); Mann, R., et al., Cell, 33:153-159 (1983); Cone, R.D. and R.C. Mulligan, Proceedings of the National Academy of Sciences, U.S.A., 81:6349-6353 (1984). Additional
- 25 details on available retrovirus vectors and their uses can be found in patents and patent publications including European Patent Application EPA 0 178 220, U.S. Patent 4,405,712, Gilboa, Biotechniques 4:504-512 (1986) (which describes the N₂ retroviral vector).
- 30 The teachings of these patents and publications are incorporated herein by reference.

Retroviral vectors are particularly useful for modifying mammalian cells because of the high efficiency with which the retroviral vectors "infect"

35 target cells and integrate into the target cell

genome. Additionally, retroviral vectors are highly useful because the vectors may be based on retroviruses that are capable of infecting mammalian cells from a wide variety of species and tissues.

5 The ability of retroviral vectors to insert into the genome of mammalian cells have made them particularly promising candidates for use in the genetic therapy of genetic diseases in humans and animals. Genetic therapy typically involves (1)
10 adding new genetic material to patient cell *in vivo*, or (2) removing patient cells from the body, adding new genetic material to the cells and reintroducing them into the body, i.e., *in vitro* gene therapy. Discussions of how to perform gene therapy in a
15 variety of cells using retroviral vectors can be found, for example, in U.S. Patent Nos. 4,868,116, issued September 19, 1989, and 4,980,286, issued December 25, 1990 (epithelial cells), W089/07136 published August 10, 1989 (hepatocyte cells), EP
20 378,576 published July 25, 1990 (fibroblast cells), and W089/05345 published June 15, 1989 and WO/90/06997, published June 28, 1990 (endothelial cells), the disclosures of which are incorporated herein by reference.

25 In order to be useful for the various techniques of gene therapy, suitable retroviral vectors require special characteristics that have not hitherto been available. A primary source of the need for these special requirements of the vector for use in the *in vivo* genetic manipulation of patient cells in gene
30 therapy is because it is usually not feasible to use retroviral vectors that require a selection for integration of the vector into the genome of "patient" cells. For example, typical retroviral vectors, e.g.,
35 MSV DHFR-NEO described in Williams, et al., Nature

310:476-480 (1984), uses neomycin resistance as a suitable marker for detecting genetically modified cells. Thus, with such neomycin resistant retroviral vectors, patients would be required to be exposed to high levels of neomycin in order to effect genetic repair of cells through *in vivo* gene therapy. Moreover, in both *in vivo* and *in vitro* gene therapy it may be undesirable to produce the gene product of the marker gene in cells undergoing human gene somatic therapy. For example, there is no therapeutic reason to produce large levels of neomycin phosphotransferase in blood cells undergoing hemoglobin gene replacement for curing a thalassemia. Therefore, it would be desirable to develop retroviral vectors that integrate efficiently into the genome, express desired levels of the gene product of interest, and are produced in high titers without the coproduction or expression of marker products such as antibodies.

Despite considerable progress in efforts to develop effective genetic therapies for diseases involving hematopoietic cells, a number of significant technical hurdles remain. First, while a variety of transduction protocols have been developed which make it possible to efficiently transfer genes into murine hematopoietic stem cells, it has not yet been possible to achieve efficient gene transfer into reconstituting cells of large animals. It is currently unclear to what extent this problem is vector related (e.g. insufficient titers, host range) or a consequence of a lack of knowledge regarding the optimal conditions for obtaining the proliferation and/or efficient engraftment of appropriate target cells. A second important technical stumbling block relates to the development of retroviral vectors possessing the appropriate signals for obtaining high level

constitutive expression of inserted genes in hematopoietic cells *in vivo*. Although a number of groups have demonstrated the expression of genes in mice reconstituted with transduced bone marrow cells, others have experienced difficulties (10-12). Overall, few general principles regarding features of vector design important for gene expression *in vivo* have emerged. In particular, because of differences in vector backbones, inserted genes, viral titers, transduction protocols, and other experimental parameters, it has been impossible to directly compare the performance of different vectors and to determine the features of vector design which most critically affect gene expression in hematopoietic cells *in vivo*. In addition, few studies have examined the ability of transferred genes to be expressed for very long periods of time (e.g. the lifetime of the transplant recipients), a clearly important goal of gene therapy for diseases involving hematopoietic cells.

20

3. SUMMARY OF THE INVENTION

The present invention is directed to a family of novel retroviral vectors capable of being used in somatic gene therapy. The retroviral vectors of the invention include an insertion site for a gene of interest and are capable of expressing desired levels of the encoded protein in a wide variety of transfected cell types.

In one aspect of the invention there is provided a retroviral vector comprising in operable combination, a 5' LTR and a 3' LTR derived from a retrovirus of interest, and an insertion site for a gene of interest, and wherein at least one of the gag, env or pol genes in the vector are incomplete or defective. The vector preferably contains a splice

35

donor site and a splice acceptor site, wherein the splice acceptor site is located upstream from the site where the gene of interest is inserted. Also, the vector desirably contains a gag transcriptional
5 promoter functionally positioned such that a transcript of a nucleotide sequence inserted into the insertion site is produced, and wherein the transcript comprises the gag 5' untranslated region. The preferred vectors of the invention are lacking a
10 selectable marker, thus, rendering them more desirable in human somatic gene therapy because a marker gene product, such as an antibiotic drug marker, will not be co-produced or co-expressed.

The gene of interest that is incorporated in the
15 vectors of the invention may be any gene which produces a hormone, an enzyme, a receptor or a drug(s) of interest.

The retroviral vectors are most suitably used in combination with certain packaging cells, as herein
20 defined, which in turn may be used in a wide variety of cell types for human or animal somatic gene therapy.

A particular preferred retroviral vector of the invention is identified herein as "MFG", as depicted
25 in Figures 2c and 3, and the plasmid containing it, and especially the plasmid MFG having the identifying characteristics of ATCC No. 68,754.

The present invention is also directed to retroviral vectors similar to those described above,
30 but further comprising a non-LTR enhancer and the alpha-globin transcriptional promoter sequence in order to control the expression of various genes of interest. This aspect of the invention specifically provides for the use of an enhancer sequence from
35 cytomegalovirus. Also provided are vectors in which

the enhancer sequence is deleted from the 3' LTR thus resulting in the inactivation of the 5' LTR upon integration of the vector into the genome. The α -globin promoter containing vector α -SGC is

- 5 specifically provided, and especially that which is depicted in Figure 4, and the plasmid containing it, and especially the plasmid α -SGC having the identifying characteristics of ATCC No. 68,755.

10 4. BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a schematic representation of a wild type murine leukemia virus (retroviral) genome.

- Figure 2 is a schematic representation of
15 retroviral vectors, each having a recombinant genome, useful in the present invention. Figure 2a is pLJ and Figure 2b is pEm, Figure 2c is MFG and Figure 2D is α -SGC.

- 20 Figure 3 is a schematic diagram of the retroviral vector MFG.

Figure 4 is a schematic diagram of the retroviral vector α -SGC.

25

Figure 5 is a histogram showing the patency after implantation into dogs of synthetic grafts lined with endothelial cells genetically augmented to express TPA.

30

- Figure 6 is a diagram of the factor VIII polypeptide. Figure 6b is a diagram of the factor VIII cDNA showing the restriction enzyme sites used in the various constructs to generate the retroviral
35 vector. Figure 6c is a diagram of the deletion

derivative of the factor VIII CDNA inserted into the retroviral vector with the deleted region shown as vertical lines. Figure 6d is an expanded diagram of the B domain deletion between the Hind III and Pst I sites. The nucleotide sequence at the junction of the heavy chain and light chain is denoted above the line and the corresponding amino acid numbers are denoted below the line.

Figure 7 is a diagram of the assembled final retroviral vector, MFG-factor VIII.

Figure 8 is a diagram of the α -SGC-LacZ recombinant retrovirus.

Figures 9(a) and 9(b) represents a schematic diagram of the construction of the MFG vector of the invention.

Figure 10 is a schematic representation of the modification of the tPA gene, the oligonucleotides used to facilitate the modification and the insertion of the modified tPA gene into the MFG vector.

Figure 11. Structure of retroviral vectors encoding human adenosine deaminase (huADA). (A) MFG-derived recombinant retroviruses. The MFG vector is derived from Mo-MuLV. The 5'-region extends to the Nar I site at position 1035 thus retaining the ψ element, Mann R. et al., Cell, 33:153-159, (1983), the splice donor (SD) and some gag coding sequence. The start codon of gag has been mutated by insertion of a Sma I linker. The 5' fragment is linked to the Nde I (+5401)- Nla III (+5780) fragment that contains the splice acceptor (SA) necessary for the generation of

the env mRNA. A point mutation (A- C) has converted the *Nla III* site into an *Nco I* site encompassing the env initiation codon where the human ADA coding sequence was inserted (from *Nco I* +74 to *Acc I* +1324 within the huADA cDNA (□). Daddona, P.E., et al., J. Biol. Chem. 259:12101-12106, (1984). The Mo-LTR/B2 vector was constructed by ligating the 1274 pb *Hind III*-*Pvu I* fragment of the PEM-ADA vector, Wilson, J.M. et al., Proc. Natl. Acad. Sci. USA 87:439-443, 10 ((1990), that contains the B2 mutation (G to A at position +160) to the *Hind III*-*Pvu I* fragment of MFG. The MPSV enhancer was cloned into MFG by replacing the *Nhe I*-*Sac I* fragment of the 3' Mo-MuLV LTR with the 385 bp corresponding fragment from the 3' MPSV-LTR 15 plasmid (kindly provided by P. Robbins, Pittsburgh, PA) to generate the MPSV-Enh construct. The MPSVE-EnhB2 was analogously derived from MPSV-Enh and PEM-ADA constructs. In the MPSV-LTR construct the 6014 bp *Ban II*-*Nhe I* fragment from the pC663neoR plasmid, 20 Ostertag, W., et al., J. gen. Virol. 67:1361-1371, (1986), has been replaced with the 2694 bp *Ban II*-*Nhe I* fragment from the MFG vector. To generate the Fr-Enh construct, the 450 bp *Nhe I*-*Kpn I* fragment of MFG was replaced with the corresponding *Nhe I*-*Kpn I* 25 fragment from the pFr-SV (X) plasmid, Holland, et al., Proc. Natl. Acad. Sci., USA, 84:8662-8666, (1987). Mo-MuLV LTR (□), MPSV sequences (□) Friend sequences (□).

(B) αG-SGC vector: The αG-SGC vector derived 30 from pHSG, bears a portion of gag and an enhancer deletion in the 3' LTR, Guild, et al., J. Virol., 62:3795-3801, (1988). In this vector, huADA expression is under the control of the human cytomegalovirus (CMV) enhancer (□) (*Spe I* +154-*Nco I* 35 +515 fragment), Boshart, M. et al., Cell, 41:521-530,

(1985), and α -globin promoter (\square) (Pst 1 -570 -NcoI +37 fragment), Braelle, F.E., Cell, 12:1085-1095, (1977).

(C) DNA analysis of NIH 3T3 cells infected with the recombinant retroviruses: After infection of NIH 3T3 cells under standard conditions (see Section 11.1, *infra*), genomic DNA was digested with *Nhe I* and analyzed by Southern blot using a huADA cDNA probe. Each lane was loaded with 10 μ g of genomic DNA. The number of proviral copy per cell is indicated under each lane as determined with the Phosphorimager. In the left lane, the copy control correspond to 1 copy per cell of the Mo-LTR vector.

Figure 12. Analysis of human ADA expression in peripheral blood cells:

(A) Analysis of hADA expression 5-7 months after BMT. The time at which blood samples were drawn is indicated in days after transplantation for each vector. hADA activity was measured by IEF (see Section 11.1., *infra*). The number directly above each sample indicates individual animals. The number of cells injected in every recipient is indicated above and extends from 2 $\times 10^5$ to 4.5 $\times 10^6$ cells. The lower band on the gel represents the activity of the murine endogenous ADA (mADA) and the upper band represents the human ADA (huADA) activity control samples were prepared from non-transplanted mice. The italic numbers indicate the mice which were examined in detail in Figure 13.

(B) Fraction of mice expressing huADA at 5-7 months after BMT. Relative ADA activity (r) represents the ratio of the intensity of human to mouse ADA enzyme bands determined on Figure 12A: with the computer densitometer. n_1 indicates the number of

mice in which $r > 1$ and n , the number of mice in which $1 < r \leq 1/4$. N represents the total number of mice analyzed.

(C) Comparison of human ADA expression in PBC 5-7
5 and 12-14 months after BMT. Blood samples were drawn at two distant time points after transplantation as indicated by the arrow and analyzed for ADA activity as described in Figure 12A. Individual mice are designated by their number (#). Arrows indicate mADA
10 and huADA activity. The relative ADA activity indicated under each sample is determined as the ratio of the intensity of the human to mouse enzyme bands. The percentage indicated in the first column represents, for each vector, the mean hADA activity
15 12-14 months after BMT compared to the original activity (100%) measured 5-7 months after BMT. (n) represents the number of mice used to calculate the mean activity.

20 Figure 13 Quantification of huADA expression in hematopoietic cell fractions 12-14 months after BMT.

(A) Analysis of huADA activity in hematopoietic cell fractions of individual mice. The animals, designated by their number, were sacrificed, cell
25 fractions were harvested and huADA activity determined by IEF in each fraction^(a). The enzymatic activity is reported in arbitrary units expressed per proviral copy per μg total protein. "0" = no detectable huADA activity; "nd" = not determined.

30 (B) Average human activity per proviral copy per μg total protein. The average huADA activity is presented for every recombinant vector in every fraction^(a). The statistical significance of the normalized differences of huADA activity between Mo-

LTR and each of the other vectors is indicated as described^(b).

(C) Average proviral copy number per cell. DNA was isolated from each cell fraction of all animals and analyzed by the method of Southern using a huADA probe. For each sample, the exact copy number was determined using the Phosphorimager, taking as a reference a cell clone known to have one copy per cell. The average proviral copy number per cell and the statistical analyses^(b) are presented for every recombinant vector in each cell fraction^(a).

(D) Average human ADA activity per μ g total protein. The average hADA activity was determined for each vector independently of the proviral copy number. The significance of the differences of hADA activity between Mo-LTR and each of the other vectors is indicated.

^(a) BM, unfractionated bone marrow; Spleen, unfractionated spleen; B Lymph, splenic B lymphocytes; T Lymph, splenic T lymphocytes; Mac, macrophages derived from BM.

^(b) Each bar marked with an asterisk (*) indicates that a distribution made of 1) pooled BM and spleen samples; 2) B lymphocytes; 3) T lymphocytes; 4) macrophages shows statistically significant difference ($P < 0.05$) when compared to the corresponding "Mo-LTR distribution" (Student-Fischer's t test).

The number of mice (n) used to calculate the means \pm SD is indicated under each vector and is derived from the mice analyzed in detail in Figure 13A.

Figure 14 provides a comparison of the DNA sequences for the Moloney murine leukemia virus ("MoMuLV") (SEQ ID NO:5), the MFG vector (SEQ ID NO:6)

and the MFG-S vector (SEQ ID NO:7). Nucleotides 320-643 of MoMuLV are shown at the top of each line. The putative CTG start codons for the cell surface gag protein and the ATG start codon for the cytoplasmic gag proteins are in enlarged letters. The gag open reading frames are denoted by underline. 'X' indicates that the nucleotide is unchanged, and a '--' indicates that a nucleotide has been deleted. The nucleotide substitutions which differentiate MGF and MFG-S are indicated by boxing. Both MFG and MFG-S have a linker insertion following the ATG of the gag ORF which is not present in MoMuLV.

Figure 15 illustrates the structure of the retroviral vector MGF-S. SA=splice acceptor, SD=splice donor, ψ =packaging signal. Note that the figure is not drawn to scale.

Figure 16 is a circular restriction map of the vector MGF-S.

Figure 17 is a DNA sequence of the 8045 BP vector MFG-S (SEQ ID NO:7).

5. DETAILED DESCRIPTION OF THE INVENTION

The subject invention provides for several retroviral vectors. The retroviral vectors provided for contain (1) 5' and 3' LTRs derived from a retrovirus of interest, the preferred retrovirus source for the LTRs is the Maloney murine leukemia virus, and (2) an insertion site for a gene of interest. The retrovirus vectors of the subject invention do not contain either a complete gag, env, or pol gene, so that the retroviral vectors are incapable of independent replication in target cells.

Preferred retroviral vectors contain a portion of the gag coding sequence, preferably the partial gag coding sequence comprises a splice donor site and a splice acceptor site, positioned such that the partial gag sequence is located in the retroviral vector so that the splice acceptor site is located closest to, and upstream from, the insertion site for the gene of interest. In a particularly preferred embodiment of the subject vectors, the transcriptional promoter is positioned such that a transcript initiated from the gag promoter contains untranslated 5' gag sequence and transcript produced from nucleic acid sequence inserted into the insertion site in the vector. Vectors of interest preferably do not contain selectable markers. A preferred embodiment of such vectors is the vector designated as "MFG".

Another aspect of the subject invention is to provide for retroviral vectors lacking functional enhancer elements in the 3' LTR, thereby inactivating the 5' LTR upon integration into the genome of target organisms.

Another aspect of the subject invention is to provide for retroviral vectors essentially as described above but instead of utilizing the gag promoter to control the expression of a gene inserted into the insertion site of the vector, a human alpha globin gene transcriptional promoter is used. The retroviral vector α -SGC is specifically disclosed.

Another aspect of the subject invention is to employ enhancer sequences not located in the LTRs in retroviral vectors using the alpha globin transcriptional promoter to increase the expression of a gene of interest. Of particular interest are vectors in which the enhancer sequence is placed upstream of the alpha globin transcriptional promoter.

Another aspect of the subject invention is to the enhancer sequence derived from a cytomegalovirus in such non-LTR enhancer containing vectors.

Another aspect of the subject invention is to
5 provide for retrovirus vector constructions containing genes for expression inserted into the insertion site in the retrovirus vector. Genes for insertion into the subject retrovirus vectors include any of a variety of hormones, enzymes, receptors or other
10 drugs. The subject invention specifically provides for the genetic constructions consisting of TPA and Factor VIII inserted (individually) into the insertion i.e., cloning sites of MFG and α -SGC.

The wild type retroviral genome has been modified
15 by Cone and Mulligan, supra for use as a vector capable of introducing new genes into cells. As shown in Figures 2, the gag, the pol and the env genes have all been removed and a DNA segment encoding the neo gene has been inserted in their place. The neo gene
20 serves as a dominant selectable marker. The retroviral sequence which remains part of the recombinant genome includes the LTRS, the tRNA binding site and the Psi packaging site. Cepko, C. et al., Cell, 37:1053-1062 (1984).

25 In addition to teaching numerous retroviral vectors containing sites for insertion of foreign genes for expression, the subject invention also provides for genetic constructions in which the retroviral vectors contain genes inserted into the
30 site for insertion i.e., foreign genes or genes for expression. Foreign genes for inclusion in the vectors of the subject invention may encode a variety of proteins. Proteins of interest include various hormones, growth factors, enzymes, lymphokines,
35 cytokines, receptors and the like. The term "foreign

genes" includes nucleic acid sequences endogenous to cells into which the retrovirus vector containing the foreign gene may be inserted. Of particular interest for use as genes for expression are those genes

5 encoding polypeptides either absent, produced in diminished quantities, or produced in mutant form in individuals suffering from a genetic disease. Additionally, it is of interest to use foreign genes encoding polypeptides for secretion from the target

10 cell so as to provide for a systemic effect induced by the protein encoded by the foreign gene. Specific foreign genes of interest include those encoding hemoglobin, interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-5, interleukin-6,

15 interleukin-7, interleukin-8, interleukin-9, interleukin-10, interleukin-11, etc., GM-CSF, G-CSF, M-CSF, human growth factor, insulin, factor VIII, factor IX, tPA, LDL receptors, tumor necrosis factor, PDGF, EGF, NGF, IL-1ra, EPO, β -globin and the like, as

20 well as biologically active muteins of these proteins. Genes for expression for insertion into retroviral vectors may be from a variety of species; however, preferred species sources for genes of interest are those species into which the retroviral vector

25 containing the foreign gene of interest is to be inserted.

The retroviral vectors of the subject invention are typically used by transfecting the nucleic acid sequences into packaging cell lines. Packaging cell

30 lines contain viral gene functions that have been deleted from the retrovirus in the course of converting it to a vector. Thus, retroviral vectors of the subject invention, either with or without genes for expression inserted into the vector insertion

35 site, may be into packaging cell lines to produce

transfected infectious virus particles containing the desired genetic construction. Ideally, packaging cell lines are capable of producing a high titer of recombinant retrovirus. Preferred packaging cell

5 lines include but are not limited to Psi-2, Psi-Am, Psi-CRIP, and Psi-CRE. Psi2 is particularly preferred for use with the retroviral vectors MFG and α -SGC.

The Psi-2 cell line described by Mulligan and coworkers was created by transfecting NIH 3T3
10 endothelial cells with pMOV-Psi, which is an ecotropic Moloney murine leukemia virus (Mo-MuLV) clone. pMOV-Psi expresses all the viral gene products but lacks the Psi sequence, which is necessary for encapsidation of the viral genome. pMOV-Psi expresses
15 an ecotropic viral envelope glycoprotein which recognizes a receptor present only on mouse (and closely related rodent) cells.

Another cell line is the Psi-am line, which are Psi-2-like packaging cell lines. These Psi-am cell
20 lines contain a modified pMOV-Psi-genome, in which the ecotropic envelope glycoprotein has been replaced with envelope sequences derived from the amphotropic virus 4070A (Hartley and Rowe, 1976, Journal of Virology, 19: 19-25). As a result, they are useful for
25 production of recombinant virus with amphotropic host range. The retrovirus used to make the Psi-am cell line has a very broad mammalian host range (an amphotropic host range) and can be used to infect human cells. If the recombinant genome has the Psi
30 packaging sequence, the Psi-am cell line is capable of packaging recombinant retroviral genomes into infectious retroviral particles (Cone and Mulligan, 1984, Proceedings of the National Academy of Sciences, USA, 81:6349-6353).

35 Two other packaging cell lines are known as

Psi-CRIP and Psi-CRE. These cell lines have been shown to be useful to isolate clones that stably produce high titers of recombinant retroviruses with amphotropic and ecotropic host ranges, respectively.

5 These cell lines are described in Danos and Mulligan, 1988, Proceedings of the National Academy of Sciences, USA, 85: 6460-6464; and in U.S. patent application Serial No. 07/239,545 filed September 1, 1988. The teachings of the reference and the patent application
10 are incorporated herein by reference. Psi-CRIP and Psi-CRE have been deposited at the American Type Culture Collection, Rockville, MD, under Accession Nos. CRL 9808 and CRL 9807, respectively, under the terms of the Budapest Treaty.

15 MFG retains two intact overlapping open reading frames or ORFs that encode the amino terminal portion of both the cell surface and cytoplasmic gag-pol polyproteins. These ORFs provide a target region for recombination events with viral structural coding
20 sequences present in the packaging cell line which could lead to the formation of replication-competent virus. In order to minimize this already remote possibility, the MFG gag ORFs can be mutagenized in such a way as to disrupt possible recombination
25 events. Thus, a preferred retroviral vector of the invention is an MFG vector having stop codons inserted downstream from the initiation codons for the cell surface cytoplasmic gag polypeptides. A particular embodiment of this MFG vector, termed MFG-S, is more
30 fully described by way of example in Section 12., *infra*.

The subject invention also includes retroviral vectors that have a gene for expression inserted into the site for gene expression. Numerous vectors

incorporating various genes are specifically described by way of example in Sections 6-12, *infra*.

In a particular embodiment of the invention, described by way of example in Section 7, *infra*, MFG
5 and α -SGC retroviral vectors carrying the gene for human tissue-type plasminogen activator (tPA) are constructed and used to efficiently transduce target endothelial cells and direct the sustained expression of high levels of tPA. In a related embodiment,
10 described by way of example in Section 9, *infra*, MFG vectors carrying the gene for human Factor VIII are constructed and used to efficiently transduce endothelial cells and direct the expression of Factor VIII.

15 The recombinant retroviral vectors of the invention are capable of achieving transduction of cells *in vivo*. For example, as described more fully in Section 10, *infra*, the vector α -SGC-LacZ effectively transduces murine vascular endothelial
20 cells *in vivo*, resulting in sustained expression of LacZ gene product *in vivo*. Thus, the invention provides gene therapy vectors and methods for the *in situ* transduction of target cells, such as, for example, vascular endothelial cells.

25 Another aspect of the invention relates to transduction of hematopoietic cells with the recombinant retroviral vectors of the invention, and to the treatment of a wide variety of hematologic diseases and disorders via gene therapy with such
30 vectors, including, but not limited to anemias, hemolytic disorders, red blood cell metabolic disorders, hemoglobinopathies, thalassemias, neutrophil function disorders, leukopenia, erythrocytosis, myeloproliferative disorders,
35 leukemias, lymphomas, eosinophilic disorders, plasma

cell disorders, blood coagulation disorders, and the like. In a specific embodiment of this aspect of the invention, described by way of example in Section 11, *infra*, various MFG-derived recombinant retroviral

5 vectors carrying a gene of interest may be constructed and used to transduce hematopoietic stem cells present in bone marrow. Cells transduced by such vectors may be used in bone marrow transplantation procedures in order to regenerate a complete hematopoietic system

10 characterized by a variety of hematopoietic cell types producing the product encoded by the transferred gene of interest. Applicants' bone marrow transplantation study results disclosed in Section 11, *infra*, indicate that a number of different MFG-derived vectors are

15 capable of transducing hematopoietic stem cells, and direct the high level expression of a desired gene product in most hematopoietic cell lineages generated from such transduced stem cells for nearly the lifetime of a bone marrow transplant recipient. The

20 results also indicate that the choice of a particular viral LTR incorporated into the vector design may influence gene expression levels.

In view of the results presented in Section 11, *infra*, the recombinant retroviral vectors of the

25 invention are clearly capable of providing for long term sustained expression of genes in hematopoietic cells derived from transduced bone marrow cells. The ability to detect significant levels of gene expression in all hematopoietic lineages at over a

30 year post-transfusion is significant, since this time approximates the normal lifespan of a murine bone marrow transplant recipient. These results also strongly suggest that in the case of previous studies which have demonstrated either the inactivity or

35 shutoff of gene expression by LTR-based vectors,

specific features of vector design other than the utilization of viral LTRs may contribute more to the observed problems in expression than previously suspected. Although the absolute magnitude of improvement of expression afforded by either the MPSV-LTR or the B2 derivatives of MFG-ADA described in Section 11, *infra*, is somewhat difficult to assess, in light of the small number of animals examined for expression in different cell lineages and the variations in expression levels observed, the data clearly suggests that those vectors offer improved expression and that the improvement appears to be general, in that it occurs in most all hematopoietic lineages.

Accordingly, the invention also provides a method of treating a hematologic disease characterized by a defective gene in a hematopoietic cell in a patient, involving the steps of isolating allogenic, HLA-identical bone marrow cells from a donor; transducing the donor bone marrow cells with a recombinant retroviral vector of the invention engineered to contain a normal gene corresponding to the defective gene at the vector insertion site; culturing the transduced donor bone marrow cells to generate a suitable population of viable cells; destroying the patient's immune system using any suitable method, such as, for example, by the administration of cyclophosphamide (i.e., 50 mg per kg per day for 4 days), or by total body irradiation alone or in combination with cyclophosphamide or other chemotherapeutic agents well known in the art; and, administering a suitable quantity of transfused donor bone marrow cells (approximately $2-6 \times 10^8$ transfused donor bone marrow cells per kilogram body weight) to the patient via any appropriate route of

administration such as, for example, by intravenous infusion, following destruction of the patients immune system.

Yet another aspect of the invention is directed to improved vascular grafts for use in vascular surgery. A major problem with synthetic vascular grafts is their tendency to induce thrombus formation in the graft area, leading to occlusion and failure of the grafts, as well as myocardial infarction and death. Synthetic vascular grafts have never achieved long-term patency comparable to autologous saphenous vein, currently the material of choice in vascular surgical procedures such as coronary bypass operations, because of this inherent thrombogenicity. The problem is more intractable with micro-vessel grafts, and in patients that do not have an available saphenous vein for the graft.

The invention provides improved endothelialized vascular grafts which resist the formation of occlusive thrombi. More specifically, the improved vascular grafts of the invention are pre-seeded with endothelial cells which have been genetically transduced by recombinant MFG retroviral vectors carrying the gene for a thrombolytic or anti-thrombotic agent, such as tissue-type plasminogen activator (tPA). Such pre-seeded vascular grafts will, by virtue of the transduced endothelial cells lining the lumen of the graft, produce locally high levels of the thrombolytic or anti-thrombotic agent, thus inhibiting thrombus formation. In a particular embodiment of this aspect of the invention, described more fully and by way of example in Section 8, *infra*, the recombinant retroviral vector MFG-tPA is used to transduce canine endothelial cells, and the transduced endothelial cells are then used to endothelialize the

luminal surface of a synthetic vascular graft prior to implanting the graft as an aortic-iliac bypass into test animals. The grafts seeded with MFG-tPA-transduced endothelial cells are capable of

5 substantially inhibiting thrombus formation relative to grafts seeded with control endothelial cells, and therefore demonstrate an improved success rate. Such improved antithrombotic vascular grafts may therefore find use in surgical procedures such as coronary

10 bypass surgery, and may eliminate the need for utilizing autologous vasculature for graft material. Various synthetic graft materials are known in the art and may be used to prepare the improved grafts of the invention, including but not limited to polymeric

15 graft materials (e.g., polytetrafluoroethylene), teflon, and the like. Preferably, the synthetic graft material is precoated with a type of fibrinolytically-inhibited fibrin glue prior to lining the lumen of the graft with the genetically modified endothelial cells.

20 For recent discussions of endothelialized synthetic vascular grafts, see, for example, Zilla et al., 194, J. Vasc. Surg. 19:540-548; and Ahlswede and Williams, 1994, Arterioscler. Thromb. 14:25-31.

Thus there is provided an improved synthetic

25 vascular graft, comprising a lining of autologous endothelial cells genetically modified to produce an thrombolytic or anti-thrombic protein such as, for example, human tissue-type plasminogen activator, on the luminal surface of the graft, wherein said

30 endothelial cells have been modified prior to implantation of the graft by transducing parental endothelial cells with a recombinant retroviral vector of the invention, engineered to contain the coding sequence for the thrombolytic or anti-thrombic protein

35 at the vector insertion site.

The retroviral vectors of the invention may be used in a wide variety of cell types, including but not limited to epithelial cells, fibroblast cells, hepatocyte cells, endothelial cells, myoblast cells, 5 astrocyte cells, lymphocyte cells, mesenchymal cells, and the like. Of particular interest are the cell types disclosed in the following patents and patent publications: U.S. Patent Nos. 4,868,116, issued September 19, 1989, and 4,980,286, issued December 25, 10 1990 (epithelial cells), PCT/US89/00422, W089/07136 published August 10, 1989 (hepatocyte cells), EP 378,576 published July 25, 1990 (fibroblast cells), and PCT/US88/04383, W089/05345 published June 15, 1989 and WO/90/06997, published June 28, 1990 (endothelial 15 cells), the disclosures of which are incorporated herein by reference.

The vectors of the subject invention find a variety of uses in the treatment of various medical conditions, including, but not limited to cancer, 20 genetically based diseases, cardiopulmonary diseases, endocrinological diseases, and the like.

The present invention will now be illustrated by the following examples, which are not intended to be limiting in any way.

25

6. EXAMPLE: CONSTRUCTION OF MFG AND α -SGC RETROVIRAL GENE THERAPY VECTORS

6.1. MATERIALS AND METHODS

6.1.1. CONSTRUCTION OF MFG VECTOR PRECURSOR

6.1.1.1. CONSTRUCTION OF pMOV-Psi

30 Plasmid pMOVPsi was constructed as follows:
Three purified DNA fragments were ligated together to construct pMOV Psi-. The first was obtained by digesting pMOV Psi+ with Xho I to completion, followed 35 by partial digestion with EcoRI. Chumakov, I. et al., Journal of Virology, 42:1088-1098 (1982). The

fragment extending from the Xho I site at 2.0 U in MuLV, through the 3' LTR, 3' mouse flanking sequence, all of pBR322, and ending at the EcoRI site was purified from an agarose gel after electrophoretic separation. Vogelstein, B. and D. Gillespie, Proceedings of the National Academy of Sciences, USA, 761:615-619 (1979). The second fragment was obtained by digestion of 6 PMOV Psi+ with Bal I to completion followed by purification of the fragment extending from the Bal I site in pBR322 through 5' mouse flanking sequence and 5' LTR to the Bal I site located at 0.7 U of MuLV. HindIII linkers (Collaborative Research) were then blunt-ligated to this fragment with T4 DNA ligase, and the fragment was digested with excess HindIII and EcoRI. The LTR- containing fragment was purified from an agarose gel after electrophoretic separation. The third fragment present in the final ligation reaction was obtained from pSV2gag/pol where the gag/pol region of MuLV had been subcloned into pSV2. Mulligan, R.C. and P. Berg, Science, 209:1422-1427 (1980). pSV2-gag/pol was digested to completion with Xho I and HindIII and the fragment extending from the HindIII site (changed from the Pst I site at 1.0 U of MuLV) to the Xho I site at 2.0 of MuLV was purified from an agarose gel following electrophoretic separation. These three DNA fragments were then mixed in equimolar amounts at a total DNA concentration of 50 ug/ml. in ligase buffer (50 mM Tris-HCl [pH 7.8]), 10 mM MgCl₂, 20 mM dithiothreitol, 1.0 mM ATP, 50 ug/ml. bovine serum albumin) and incubated with T4 DNA ligase for 18 hr. at 15 C. E. coli HB101 was transfected with the ligated DNA, and ampicillin resistant transfectants were obtained. The plasmid DNA obtained from a number of transformants was screened for the desired structure by digestion

with appropriate restriction endonucleases and electrophoresis through agarose gels. Davis, R.W. et al., Methods in Enzymology, 65:404-411 (1980).

Cell lines containing the Psi mutant stably integrated into the chromosome were made by 5 cotransfection of pMOV-Psi and pSV2gpt, a SV40 hybrid vector capable of XG PRT expression. Mulligan, R.C. and P. Berg, Science, 209:1422-1427 (1980). Cells from gpt+ colonies obtained in this way were cloned 10 and established into three lines: Psi-1, Psi-2, and Psi-3.

6.1.1.2 CONSTRUCTION OF pLJ

The characteristics of the pLJ vector have been 15 described in Korman, A.J. et al., Proceedings of the National Academy of Sciences, USA, 84:2150 (1987). This vector is capable of expressing two genes: the gene of interest and a dominant selectable marker, such as the neo gene. The gene of interest is cloned 20 in direct orientation into a BamHI/SmaI/SalI cloning site just distal to the 5' LTR, while, the neo gene is placed distal to an internal promoter (from SV40) which is farther 3' than is the cloning site (is located 3' of the cloning site). Transcription from 25 pLJ is initiated at two sites: 1) the 5' LTR, which is responsible for expression of the gene of interest and 2) the internal SV40 promoter, which is responsible for expression of the neo gene. The structure of pLJ is represented in Figure 2a.

30 Vector pLJ is represented in Figure 2a. In pLJ, the genetic material of interest is inserted just following the 5' LTR. Expression of this genetic material is transcribed from the LTR and expression of the neo gene is transcribed from an internal SV40 35 promoter.

6.1.1.3. CONSTRUCTION OF pEm

In this simple vector, the entire coding sequence for gag, pol and env of the wild type virus is replaced with the gene of interest, which is the only gene expressed. The components of the pEm vector are described below. The 5' flanking sequence, 5' LTR and 400 bp of contiguous sequence (up to the BamHI site) is from pZIP. The 3' flanking sequence and LTR are also from PZIP; however, the ClaI site 150 bp upstream from the 3' LTR has been ligated with synthetic BamHI linkers and forms the other half of the BamHI cloning site present in the vector. The HindIII/EcoRI fragment of pBR322 forms the plasmid backbone. This vector is derived from sequences cloned from a strain of Moloney Murine Leukemia virus. An analogous vector has been constructed from sequences derived from the myeloproliferative sarcoma virus. The structure of pEm is represented in Figure 2b.

Vectors without a selectable marker can also be used to transduce a variety of cell types, such as endothelial cells with genetic material of interest. Such vectors are basically simplifications of the vectors previously described, in which there is such a marker. Vector pEm is represented in Figure 2b; as represented, the main components of the vector are the 5' and 3' LTR, and the genetic material of interest, inserted between the two LTRs.

6.1.2. CONSTRUCTION OF THE MFG VECTOR

The MFG vector having the identifying characteristics of ATCC accession No. 68754 is derived from the pEM vector but contains 1038 base pairs of the gag sequence from MMLV to increase the encapsulation of recombinant genomes in the packaging cell lines, and 350 base pairs derived from MOV-9

which contains the splice acceptor sequence and transcriptional start. An 18 base pair oligonucleotide containing NcoI and BamHI sites directly follows the MOV-9 sequence and allows for the convenient insertion of genes with compatible sites. The MMLV LTR controls transcription and the resulting mRNA contains the authentic 5' untranslated region of the native gag transcript followed directly by the open reading frame of the inserted gene. The structure of MFG is represented in Figure 2c. A more detailed map of MFG is provided in Figure 13. Details for the construction of MFG are provided in Figures 9(a) and 9(b).

MFG was constructed by ligating the 5' LTR containing XhoI/NdeI fragment of the half-GAG retroviral vector (half-GAG is described in Bender, *et al.*, *J. Virol.* 61:1639-1646) to an XhoI/BamHI H4 histone promoter fragment. Retroviral vector pEM was digested with NdeI and BamHI, and the 3' LTR containing fragment was ligated to the halfGAG fragment already ligated the H4 fragment so as to produce an intermediate retrovirus vector containing 2 LTRs in the proper orientation and also containing the H4 fragment within the viral portion of the vector. The intermediate vector was then linearized by digestion with NdeI and the NdeI site in the pB322 portion of the vector was filled in by polymerase and destroyed by ligation. The vector was subsequently digested with XhoI and the XhoI site was joined to NdeI linker. The vector was subsequently cleaved with BamHI and the large fragment containing both LTRs and the pBR322 sequence) was purified.

A linker having XhoI and BamHI and having the following sequence:

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CTAGACTGCCATGGCGCG

- 30 -

TGACGGTACCGCGCCTAG

was synthesized and ligated to both the BamHI site on the cleared intermediate vector and an NdeI/XbaI fragment from pMOV9 [containing a splice acceptor site next to the NdeI edge] so as to form a circular vector, MFG as illustrated in Figures 2c, 3 and 9(a) to 9(b). The plasmid containing vector MFG has been deposited with the American Type Culture Collection and it has accession number 68,754.

10

6.1.3. CONSTRUCTION OF THE α -SGC VECTOR

The α -SGC vector (ATCC accession number 68755) utilizes transcriptional promoter sequences from the α -globin gene to regulate expression of the tPA gene.

15 The 600 base pair fragment containing the promoter element additionally contains the sequences for the transcriptional initiation and 5' untranslated region of the authentic α -globin mRNA. A 360 base pair fragment which includes the transcriptional enhancer from cytomegalovirus precedes the α -globin promoter and is used to enhance transcription from this element. Additionally, the MMLV enhancer is deleted from the 3' LTR. This deletion is transferred to the 5' LTR upon infection and essentially inactivates the

25 transcriptional activating activity of the element. The structure of α -SGC is represented in Figure 2d. A more detailed description of α -SGC is provided in Figure 4. A plasmid containing the α -SGC vector has been deposited with the American Type Culture

30 Collection and it has accession number 68,755. The following examples provide examples of using the retroviral vectors of the invention using endothelial cells. It will be understood that other cell types are suitable as well, including without limitation

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epithelial cells, fibroblast cells, hepatocyte cells and others.

7. **EXAMPLE: USE OF MFG AND α -SGC VECTORS TO AFFECT INCREASED EXPRESSION OF HUMAN TISSUE PLASMINOGEN ACTIVATOR IN ENDOTHELIAL CELLS**

Tissue plasminogen activator (tPA) is a protein normally secreted by endothelial cells that promotes fibrinolysis of blood clots. Recombinant retroviral vectors encoding human tPA were constructed and used to transduce canine endothelial cells in order to demonstrate the enhanced delivery of a therapeutically relevant protein from transduced endothelial cells.

7.1. **MATERIALS AND METHODS**

7.1.1. **CONSTRUCTION OF MFG AND α -SGC VECTORS ENCODING tPA**

The modifications of the tPA gene for cloning into the recombinant retroviral vectors are shown in Figure 10. The coding sequences of human uterine tPA were contained within a Sal I DNA fragment of a pUC-based plasmid obtained from Integrated Genetics Inc. Framingham MA. The Sal I fragment was derived by placing Sal I linkers at the SfaN I site at base pair 6 and the Bgl II site at base pair 2090 of the original cDNA. The coding sequences extends from base pair 13 to base pair 1699.

From this original clone a fragment that could be cloned directly into the MFG and α -SGC vectors described in Section 6.1, supra. The Sal I fragment was first converted to a Bam HI fragment by the addition of synthetic Bam HI linkers and then digested with the restriction enzyme Bgl II to yield a 109 base pair BamHI to BglII fragment and a 1975 base pair Bgl II to Bam HI fragment. To recreate the missing 100 base pairs of tPA coding sequences and the

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translational start codon, two 104 base pair oligonucleotides were chemically synthesized and annealed to create a fragment with an Nco I site at the 5' end and a Bgl II site at the 3' end. This oligonucleotide was ligated onto the Bgl II site of the partial 1975 base pair tPA gene to create a 2079 base pair tPA gene with the identical coding sequence of the original molecule, but which can be easily obtained as an Nco I to Bam HI fragment. It was inserted directly into the MFG and α -SGC vectors (the resulting vectors were given ATCC accession numbers 68727 and 68729, respectively). These manipulations were performed by standard molecular biological techniques (Molecular Cloning -A laboratory Manual, T. Maniatis, E.F. Frisch, and J. Sambrook), and are diagrammed in Figure 2.

7.1.2. PREPARATION OF MFG-tPA AND α -SGC-tPA PRODUCER CELL LINES

Cell lines producing recombinant virus encoding MFG-tPA and α -SGC-tPA were made from the Psi packaging cell line of Danos and Mulligan capable of producing recombinant retrovirus of amphotrophic host range [Proc. Natl. Acad. Sci. U.S.A. 85:6460 (1988)]. 10 ug of the specified DNAs and 1 ug of the plasmid pSV2neo were co-precipitated and transfected onto the packaging cells by standard calcium phosphate transfection procedures. Stably transfected clones were isolated after growth for 14 days in selective media containing 800 ug/ml G418. 24 hour culture supernatants were obtained from confluent monolayers of individual clones and used to infect NIH 3T3 cells.

The culture supernatants were removed after 24 hours exposure, and the 3T3 cells were refed with normal media and allowed to grow for an additional 72 hours. Fresh media was placed on these cells for 6

- 33 -

hours and these supernatants were assayed for human tPA with a commercially available ELISA specific for human tPA (Immunobind-5, American Diagnostica Inc., N.Y., N.Y.) From this screen, clones of the packaging
5 cell line producing either the MFG-tPA recombinant virus or the α -SGC-tPA recombinant virus were selected and designated MFG 68 and α -SGC 22, respectively.

7.1.3. TARGET ENDOTHELIAL CELLS AND TRANSDUCTION
WITH MFG-tPA AND α -SGC-tPA VECTORS

10 Canine endothelial cells were isolated from 10 cm segments of the external jugular vein by collagenase digestion as described [T.J. Hunter S.P. Schmidt, W.V. Sharp, and (1983) Trans. Am. Soc. Artif. Intern.
15 Organs 29:177]. The cells were propagated on fibronectin-coated tissue culture dishes in M199 media containing 5% plasma-derived equine serum, 50 ug/ml endothelial cell growth factor, and 100 ug/ml heparin. Purity of the cell cultures was determined by
20 immunohistochemical assay for the presence of Von Willebrands Factor and the absence of smooth muscle cell specific α -actin.

The day before transduction, the endothelial cells were seeded at 5.5×10^3 cells/cm² in medium
25 without heparin. The following day, the endothelial cells were exposed for 24 hours to supernatants containing recombinant virus derived from each producer cell line to which was added 8 ug/ml polybrene. The viral supernatants were removed, the
30 cells feed with normal media and growth was allowed to proceed for an additional 48 hours before analysis.

High molecular weight genomic DNA and total RNA were isolated from cultures of endothelial cells by standard techniques (Molecular Cloning-A Laboratory
35 Manual, T. Maniatis, E.F. Fritsch, and J. Sambrook). The DNA and RNA were analyzed by

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hybridization analysis with a ^{32}P -labeled DNA probe prepared from the entire tPA cDNA fragment. Standard techniques were used for electrophoretic separation, filter transfer, hybridization, washing, and ^{32}P -
5 labeling (Molecular Cloning-A Laboratory Manual T. Maniatis, E.F. Fritsch, and J. Sambrook). The production of human tPA in transduced canine endothelial cells was demonstrated with a species specific immunocytochemical stain. Transduced cells
10 were fixed in 3% formaldehyde for 10 minutes at room temperature and then permeabilized in 0.1% Triton X-100 for 5 minutes. The fixed cell monolayer was then incubated sequentially with a murine monoclonal antibody to human tPA, with an alkaline phosphatase
15 conjugated goat anti-mouse antibody, and finally with a color reagent specific for alkaline phosphatase. This procedure specifically stains those cells expressing human tPA and can be visualized by conventional light microscopy. In addition, tPA
20 secretion from transduced cells was determined from confluent cell monolayers. Fresh media was placed on the cells for 6 hours, removed and clarified by centrifugation, and the amount of human tPA determined with a commercially available ELISA (Immunobind-5,
25 American Diagnostics).

7.2. RESULTS

The efficiency of the transduction process is shown by immunocytochemical stain of a population of
30 cells mock transduced or transduced with MFG-tPA. As shown in Figure 5, after a single exposure of the cells to a viral supernatant harvested from MFG 68, essentially all of the cells are synthesizing human tPA as opposed to none of the cells in the control.

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This was achieved without selection of any type for transduced cells.

An immunological assay was conducted to determine the amount of tPA that was being secreted from transduced cultures. As shown below, cells transduced with recombinant virus from either MFG 68 or α -SGC 22 secreted large amounts of human tPA. Under similar conditions, human endothelial cells in culture typically secrete approximately 1 ng of tPA [Hanss, M., and D. Collen (1987) J. Lab. Clin. Med. 109: 97-104].

TABLE I

Cells	ng tPA/10 ⁶ cells/6hrs.
uninfected K9 EC	0.0
MFG 68 K9 EC	150.1
α -SGC 22 K9 EC	302.8

As a further confirmation that the endothelial cells had been transduced with recombinant virus from MFG 68 and α -SGC 22, DNA and RNA was isolated from transduced cells and analyzed by hybridization to a radiolabeled tPA gene. An autoradiogram of the DNA analysis was performed. No hybridization was detected in the uninfected controls, but single hybridizing species of the appropriate molecular weight was seen in the cells infected with the two recombinant vectors. This demonstrates that the genetic information has been transferred to the genome of these transduced cells.

Hybridization analysis of total RNA isolated from these cells confirms the protein and DNA results. Again no hybridization was detected in the control cells but in the RNA derived from the transduced cells

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hybridizing bands of the appropriate sizes can be seen. RNA from the MFG 68 and α -SGC 22 recombinant virus producing cells is also shown as controls.

5 8. **EXAMPLE: IN VIVO ANTITHROMBOTIC ACTIVITY OF VASCULAR GRAFTS SEEDED WITH MFG-tPA-TRANSDUCED ENDOTHELIAL CELLS**

8.1. **MATERIALS AND METHODS**

Endothelial cells were enzymatically harvested from external jugular veins of adult female mongrel
10 dogs that weighed 20-25 kg and cultured in the laboratory and analyzed for purity as described in Example 7, supra. One half of the cells isolated from each animal were transduced by two exposures to supernatants from the MFG 68 cell line producing the
15 MFG-tPA recombinant virus as described in the previous section. The other half were mock transduced. Growth curves conducted on each population showed no difference in growth characteristics. ELISA measurements were made on culture supernatants derived
20 from each batch of transduced cells to assure that tPA was being secreted from the augmented cells. These cells were then propagated in the laboratory for approximately one week to obtain sufficient numbers of cells.

25 For each animal from which cells had been isolated, two vascular grafts made of expanded Teflon (W.L., Gore and Associates, Inc. Flagstaff, AZ) were seeded with cells. One graft was seeded with mock transduced cells, and the other with cells transduced
30 to secrete high levels of tPA. Each graft, measuring 0.4 cm x 14 cm, was precoated with 1.5 ug/cm² fibronectin (Sigma Chemical Corp., St. Louis MO), and then seeded with 2200,000 endothelial cells/cm. The grafts were then incubated for an additional 72 hours
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in culture. Prior to implant the ends were cut off each graft and checked to assure cell coverage.

The same dogs from which the cells had been harvested were anesthetized and 10 cm segments of the seeded grafts were implanted as aorta-iliac bypasses. Each dog received two contralateral grafts; one seeded with control cells and the other seeded with cells that had been transduced to secrete high levels of tPA. Following implantation the performance of the grafts was monitored daily with a B-mode scanner which locates the graft with ultrasound and assesses blood flow through the graft by Doppler measurements (Accuson, Inc.). No drugs to reduce thrombus formation were administered to the animals.

15

8.2. RESULTS

The results of graft performance in 6 different animals were analyzed. The results are indicated in Figure 5. The implant model described above is an extremely stringent one and leads to rapid graft failure by occlusive clot formation. Normal graft function is denoted by solid bar, and a graft which is failing but still functioning by a striped bar. In the first animal, the control graft and the graft lined with transduced cells secreting enhanced levels of tPA (experimental) failed due to clot formation 24 hours after implant. In all of the other five animals, the graft lined with transduced cells secreting enhanced levels of tPA functioned longer than the graft with cells which had only been mock transduced. This difference varied from 24 hours to several months. These results demonstrate that a therapeutic effect can be achieved in vivo with MFG-transduced endothelial cells.

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9. EXAMPLE: USE OF MFG VECTOR TO PRODUCE HUMAN
FACTOR VIII IN ENDOTHELIAL CELLS

9.1. MATERIALS AND METHODS

9.1.1. CONSTRUCTION OF MFG/FACTOR VIII VECTOR

5 Endothelial cells were genetically augmented to
produce human factor VIII by transducing cells with a
retroviral vector, MFG, containing a modified human
factor VIII gene (ATCC accession no. 68726). The
10 modified factor VIII cDNA contains all of the coding
sequences for the A1, A2, A3, C1, and C2 domains,
however the B domain is deleted from amino acids 743
to 1648. The removal of the B domain and the
insertion of the modified factor VIII gene into the
retroviral vector MFG is described in detail below and
15 depicted in Figure 7.

A full-length cDNA without the 5' and 3'
untranslated sequences was obtained in a plasmid
vector inserted between the restriction sites Nco I
(5') and Xho I (3'). For removal of the B domain, the
20 factor VIII cDNA was subcloned into a plasmid vector
in 4 fragments spanning the sequences on both the 5'
and 3' sides of the B domain. The first fragment of
the factor VIII cDNA was subcloned between the
restriction sites Sal I and Pst I in the plasmid
25 vector pUC 9. The plasmid vector was cut with Sal I
and Pst I and the 5' phosphates were removed using
calf intestinal phosphatase. A 1591 base pair Xho I
(nucleotide 7263) to Nde I (nucleotide 5672) fragment,
and a 359 base pair Nde I (nucleotide 5672) to Pst I
30 (nucleotide 5313) fragment from the full-length cDNA
were isolated and ligated with the Sal I/Pst I
digested plasmid vector.

To remove the majority of the sequences encoding
the B domain which joins amino acids 742 to 1649 in
35 the same translational reading frame, 4
oligonucleotides were synthesized with a 5' Hind III

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site and a 3' Pst I site covering 168 base pairs. The oligonucleotides extend from the Hind III site at nucleotide 2427 which encodes amino acid 742 followed by amino acid 1649 which is the first amino acid of the activation peptide of the light chain through to the Pst I site at nucleotide 5313. The plasmid vector pUC 9 was digested with the restriction enzymes Hind III and Pst I, and the 5' phosphates were removed using calf intestinal phosphatase. The oligonucleotides were synthesized as 4 separate strands, kinased, annealed and ligated between the Hind III site and the Pst I site of the plasmid vector.

The subcloned Hind III/Pst I oligonucleotide was juxtaposed to the Pst I/ Xho I fragments in a plasmid vector pUC F8. To generate this plasmid, a new polylinker was inserted into a pUC 9 plasmid backbone with the new polylinker encoding the restriction enzyme sites 5' Sma I-Bam HI-Xho I-Pst I-Hind III-Asp 718-Nco I-Hpa I 3' used. The plasmid vector was digested with the restriction enzymes Bam HI and Hind III, and the 5' phosphates were removed with calf intestinal phosphatase. A partial Pst I/ Bam HI digest of the Pst I/Xho I subclone was used to isolate the 3' terminal factor VIII fragment, and a Pst I/Hind III digest of the subcloned oligonucleotides was used to isolate the heavy and light chain junction fragment. They were ligated into the plasmid vector pUC FB between the BamHI and Hind III sites.

This subclone containing the factor VIII sequences between nucleotides 2427 and 7205 was digested with Asp 718 and Hind III, and the 5' phosphates were removed using calf intestinal phosphatase. A fragment encoding factor VIII between the restriction enzyme sites Asp 718 (nucleotide 1961)

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and Hind III (nucleotide 2427) was isolated and ligated into the plasmid vector to generate a subclone (pF8 3' delta) containing the factor VIII sequences from nucleotide 1961 through to the translational stop codon at nucleotide 7205.

The construction of the retroviral vector containing the modified factor VIII gene was carried out by inserting the factor VIII gene between the restriction sites Nco I and Bam HI of the retroviral vector MFG. The factor VIII subclone pF8 3' delta was digested with Sma I and converted to a BglII site using an oligonucleotide linker. An Asp 718/Bgl II - fragment was isolated from the 3' factor VIII subclone, and a 5' factor VIII fragment containing the ATG for initiation of translation was isolated as an Nco I (nucleotide 151)/Asp 718 fragment (nucleotide 1961). The retroviral vector MFG was digested with Nco I and Bam HI, and the 5' phosphates were removed using calf intestinal phosphatase. The factor VIII fragments were ligated into the retroviral vector yielding the final factor VIII retroviral construct, see Figure 6.

9.1.2. PREPARATION OF MFG/FACTOR VIII PRODUCER CELL LINES

The cell line producing the retroviral particles was generated by transfection of the retroviral vector MFG/factor VIII into equal numbers of ecotropic packaging cells Psi CRE and amphotropic packaging cells CRIP as described by Bestwick et al. (Proc. Natl. Acad. Sci. USA 85:5404-5408 (1988)). To monitor the extent of superinfection taking place between the 2 host ranges of packaging cells, the production of biologically active factor VIII was measured using the Kabi Diagnostica Coatest for Factor VIII, Helena Laboratories, Beaumont, Texas and the

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production of viral RNA was measured by an RNA dot blot analysis. At 21 days post transfection, the mixture of transfected packaging cells was co-cultivated with the amphotropic packaging cell line

5 Psi CRIP-HIS. The CRIP HIS packaging cell line is a variant of the previously described CRIP packaging cell line. The CRIP HIS packaging cell line is identical to the Psi CRIP packaging cell line except that the retroviral envelop gene was introduced into

10 the cell by cotranfection with pSV2-HIS plasmid DNA, a different dominant selectable marker gene. The packaging cell lines were cultured at a 1:1 ratio for isolation of a homogeneous amphotropic retroviral stock of transducing particles. The superinfection of

15 the amphotropic packaging cell line CRIP HIS has led to the generation of a stable cell line, HIS 19, which produces recombinant retrovirus that efficiently transduce the modified human factor VIII gene. Antibiotic selection of the retroviral introducing

20 cell line was not required to isolate a cell line which produces high-titer recombinant retrovirus. The genomic DNA of the cell line has been characterized by Southern blot hybridization analysis to determine the number of integrated copies of the retroviral vector

25 present in the producer cell line. The copy number in the retroviral producing cell line is approximately 0.5, therefore on average 50% of the CRIP-HIS packaging cells contain a copy of the retroviral vector with the modified factor VIII gene. The

30 retroviral vector and the modified factor VIII gene are intact without any deletions or rearrangements of the DNA in the packaging cell line. The copy number of the retroviral vector remains constant with the continuous passage of the retroviral producing cell

35 line. For obtaining the highest titer of recombinant

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retrovirus, HIS 19 was carried 3 passages in selective histidine minus media followed by 4 passages in completed DMEM media. For the generation of retroviral particles, HIS 19 was seeded at 5×10^5 - 1×10^6 cells in a 10 cm cell culture dish. At 48 hours postseeding, approximately 70% confluency, fresh medium (DMEM + 10% calf serum) was added to the plates for collection 24 hours later as the source of recombinant retrovirus for transduction.

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9.1.3. ENDOTHELIAL CELL TRANSDUCTION

The modified factor VIII gene was transduced into canine endothelial cells isolated from the jugular vein. The endothelial cells were seeded at 3×10^5 cells per 10 cm. dish in complete M199 medium with 5% plasma derived serum (Equine), 100ug/ml heparin, and 50ug/ml endothelial cell growth factor for 4-6 hours. The cells were then incubated overnight in M199 medium with 5% plasma derived serum, and 100ug/ml endothelial cell growth factor overnight without heparin which adversely affects the efficiency of the transduction process. Cells were exposed to the fresh viral supernatant plus polybrene (8 ug/ml) for 24 hours. After removal of the viral supernatant, the cells were put into M199 medium with 5% plasma derived serum, 100ug/ml endothelial cell growth factor to grow to approximately 70-80% confluence. At that time, the medium was changed to M199 medium with 5% heat inactivated fetal bovine serum (heated at 66°C for 2 hours) , and 50 ug/ml of ECGF. Following a 24 hr. incubation, the medium was collected and assayed for biological active factor VIII by the Kabi Coatest.

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9.2. RESULTS: IN VITRO TRANSDUCTION OF ENDOTHELIAL CELLS

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With this retroviral producing cell line, between 50% and 75% of the endothelial cells were transduced as determined by Southern blot analysis. The factor VIII gene can be transduced at this frequency with a single exposure to the recombinant retrovirus, and without antibiotic selection of the transduced cells. The transduced endothelial cells contain an intact copy of the recombinant retroviral genome and the modified factor VIII gene without any deletions or rearrangements. The rate of production of biologically active factor VIII from the genetically augmented endothelial cells was 400ng/5x10⁶ cells/24 hrs.

15 10. EXAMPLE: IN VIVO TRANSDUCTION OF ENDOTHELIUM

Using standard stocks of recombinant retrovirus made as described in the previous examples, data demonstrating the *in vivo* transduction of endothelial cells has been generated as described herein. The approach is based on the previously published observation (Reidy MA, Schwartz SM, Lab Invest 44:301-308 (1981)) that a defined injury to an artery surface removes a small strip of endothelial cells and this denuded area heals within seventy-two hours by proliferation and in growth of new endothelial cells from the edge of the defect. Cell division is a requirement for effective transduction by recombinant retroviruses and the injury of the endothelium with a wire is one of potentially many methods to induce endothelial cell proliferation. Our method uses Reidy's technique of defined injury to induce endothelial cell proliferation, then exposes the proliferating cells directly to supernatants containing recombinant retroviral vectors. Our initial experiments document the ability of this

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method to successfully transduce endothelial cells in situ, thus potentially avoiding the necessity of tissue culture techniques for the successful introduction of new genetic sequences.

5 This method requires two surgical procedures, the first procedure injures the blood vessel surface (here described for the right iliac artery) and induces the proliferation of endothelial cells. The second procedure delivers recombinant retrovirus to the cells
10 undergoing replication on the vessel surface, while preventing the flow of blood from the proximal arterial tree while the proliferating cells are exposed to retroviral particles. For simplicity of performance the procedure is described for iliac
15 arteries.

10.1. MATERIALS AND METHODS

To demonstrate *in vivo* gene transfer, we used the marker gene concept published in 1987 (Price J, Turner
20 D. Cepko C. 1987 Proc. Natl. Acad. Sci. USA 84:156160.) with an improved vector based on the α -SGC vector (Figures 2d and 4). The lacZ gene encoding beta-galactosidase was inserted into the α -SGC vector to generate the α -SGC-LacZ vector which is represented
25 in Figure 8. This recombinant construct was transfected into the t Crip packaging cell line and a clone of t Crip cells producing high titers of the α -SGC-LacZ recombinant retrovirus were isolated as described in Example 9, *supra*. Stocks of the α -SGC-
30 LacZ recombinant retrovirus were used for *in vivo* transduction.

The experimental animals (rabbits) were anesthetized (ketamine/xylazine), both groins were shaved and prepped, and the animals positioned on an
35 operating table. Through bilateral vertical groin

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incisions the common, superficial, and profunda femoral arteries were exposed. On the right (the side to be injured) small branches off the common femoral artery were ligated to insure that outflow from the isolated arterial segment would only occur through the internal iliac artery. If necessary, the inguinal ligament was divided and the vessel followed into the retroperitoneum to assure complete control of all side branches. The right superficial femoral artery (SFA) was ligated with 3-0 silk approximately 1.5 cm below the profunda take-off, control of the SFA was obtained at the SFA/profunda junction, and a transverse arteriotomy created. A fine wire (the stylet of a 20 gauge Intracath was used), doubled upon itself to provide springiness to assure contact with the vessel wall, was passed up the common femoral and iliac artery retrograde to produce the defined injury described by Reidy et al. The wire was removed, a 20 gauge angiocath was inserted in the arteriotomy and secured to the underlying muscle for immediate access at the next surgical procedure. The incisions were closed in layers and the animals allowed to recover.

Twenty-four hours later a recombinant virus containing supernatant harvested from a crup producer of the α -SGC-LAC-Z vector and supplemented with polybrene to a final concentration of 8 ug/ml was used for *in vivo* transduction. The animals were again anesthetized and both incisions reopened in a sterile environment. To obtain control of the right iliac vessels above the area that had been injured with no disturbance to the previously denuded right iliac vessel, a #3 FogartyTM balloon embolectomy catheter was inserted through an arteriotomy in the left superficial femoral artery, passed to the aortic bifurcation and the balloon inflated to interrupt

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blood flow. The right profunda femoris artery was occluded. The supernatant (10 ml) containing the recombinant retrovirus was introduced by hand injection through the angiocath previously placed in the right SFA. The supernatant flowed in a retrograde fashion from the right common femoral to the right external iliac and into the right internal iliac artery. By leaving the right internal iliac artery open outflow for the supernatant was allowed and a full 10 ml of supernatant could be instilled. In the experiments performed to date the supernatants have been exposed to the vessel wall for periods of four to eight minutes. The catheters from the left and right sides were then removed, hemostasis obtained, and the incisions closed.

Ten to fourteen days later animals were anesthetized prior to sacrifice. After anesthesia and prior to exposure, patency was assessed by direct palpation of the distal vessel. The infra-renal aorta and inferior vena cava were surgically exposed, cannulated, and the vessels of the lower extremity flushed with heparinized Ringer's lactate (2 U/ml) at physiologic pressure (90 mmhg.) A lethal dose of nembutal was administered and the arteries perfusionfixed in situ in 0.5% glutaraldehyde in 0.1 M cacodylate for 10 minutes. The aorta and both iliac arteries were excised in continuity and rinsed in phosphate buffered saline (PBS) with 1mM MgCl₂. The vessels were then stained for lacZ activity by incubation in the x-gal substrate for 1-1.5 hours at 37°C. When the reaction was complete, the x-gal solution was washed away and replaced with PBS.

10.2. RESULTS

Two experiments have been completed with this protocol. Both experiments demonstrated successful in vivo transduction as shown by the in situ expression of the lacZ gene product in cells on the surface of the artery as visualized by the selective intense blue staining in a cytoplasmic pattern. A line of intensely stained blue cells consistent with the pattern of injury and proliferation described by Reidy et al. is found on the surface of a segment of the external illiac artery injured with a wire, exposed to α -SGC-LacZ recombinant retrovirus, fixed and stained for lacZ activity.

11. **EXAMPLE: IN VIVO EXPRESSION OF HUMAN ADENOSINE DEAMINASE BY HEMATOPOIETIC STEM CELLS TRANSDUCED WITH SEVERAL RECOMBINANT MFG-ADA RETROVIRAL VECTORS**

Several different MFG-based vectors encoding the same gene product, human adenosine deaminase (huADA) and the same vector backbone, yet differing specifically in transcriptional control sequences were constructed and evaluated for their capacity to efficiently transduce murine hematopoietic stem cells. Transduced stem cells were subsequently used in bone marrow transplantation experiments, and the long term vector-mediated expression of huada by various hematopoietic cells following transplantation.

11.1 **MATERIALS AND METHODS**

11.1.1. **RECOMBINANT RETROVIRUS VECTORS AND GENERATION OF RETROVIRUS-PRODUCING CELLS**

All recombinant retrovirus constructs but the α G-SGC vector are based on the retroviral vector MFG (MFG/Mo-LTR) described supra and depicted in Figure 11A. All new structures generated were verified by DNA sequencing. Retroviral producer cell lines were generated by co-transfecting each retroviral plasmid

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with the plasmid pSV2-Neo into the amphotropic packaging cell line Ψ CRIP as previously described (Danos and Mulligan, 1988, Proc. Natl. Acad. Sci. USA, 85:6460-6464). Cell-free supernatant was harvested
5 after 10 days of selection in the presence of G418 at 1 mg/ml (Gibco BRL, Grand Island, NY) and used to infect Ψ CRE cells. An α G-SGC producer was isolated after direct transfection into CRE cells. Twenty five clones per vector obtained by limiting dilution were
10 screened for high-titer by Southern blot analysis. Titration was performed by infecting 5×10^5 NIH 3T3 cells with 0.5 ml of a 24 hours supernatant from the virus-producing clones in the presence of 8 μ g/ml of polybrene (Sigma Chemical Co., St Louis, MO). Genomic
15 DNA was extracted for Southern blot analysis to quantitate the number of proviral copies integrated in the target population. To verify the presence of the B2 mutation in the selected virus-producer cell lines and its transmission to infected 3T3 cells, PCR
20 primers corresponding to Mo-MuLV nucleotides 72-92 and 470-490 were used to amplify a fragment of 400bp which was then sequenced (fmolTM DNA Sequencing System, Promega, Madison, WI). Supernatants from Ψ CRE virus-producing cells and plasma from transplant recipients
25 were tested for the presence of replication-competent virus based on a mobilization assay (Wilson et al., 1990, Proc. Natl. Acad. Sci. USA 87:439-443). In our study, the assay was modified to detect recombinant retroviral genomes expressing *Salmonella typhimurim*
30 histidinol dehydrogenase as described (Hartman and Mulligan, 1988, Proc. Natl. Acad. Sci. USA 85:8047-8051).

Genomic DNA was prepared as described (Seif et al., 1991, Methods Mol. Cell. Biol. 2:216-217) and
35 digested with *Nhe* I or *Ec*1136 II (for proviral

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structure) and Nco I or Bam HI (for proviral integration patterns). Hybridization filters were probed with a 721 bp ³²p- labeled BamH I-Bgl II fragment of the hADA cDNA (Multiprime DNA labeling system, Amersham). The copy numbers were determined on a Phosphorimager (Fuji Bio-Imaging, Fuji Medical Systems, Stamford CT) relative to the intensity of bands generated in a cell line infected with a single copy of the provirus. To account for uneven loading, the band signal was normalized to the murine endogenous ADA band.

11.1.2. TRANSDUCTION OF MURINE BONE MARROW CELLS WITH RECOMBINANT RETROVIRAL VECTORS AND TRANSPLANTATION OF TRANSDUCED CELLS INTO MICE

Bone Marrow (BM) cells were obtained from the tibias and femurs of C57BL/6J male mice as previously described (Wilson et al., supra) (Jackson Laboratories, Bar Harbor, ME), 6 days after intravenous injection of 150 mg/kg body weight of 5-fluorouracil. Transduction was achieved by coculturing BM cells for 48 hr on a monolayer of Ψ CRE producers in the presence of 10% (vol/vol) WEHI-3B supernatant and 4 μ g/ml polybrene. Nonadherent cells were harvested and 2×10^5 to 4.5×10^6 viable cells per mouse were injected via the tail vein into total body irradiated (11 Gy) syngeneic female hosts. Transplanted mice were sacrificed 12 to 14 months after transplantation and samples of peripheral blood, EM, spleen, spleen-derived B and T lymphocytes, and BM-derived macrophages were analyzed for the presence of provirus and enzyme expression. B and T cells were harvested after 72 hr stimulation with 10 μ g/ml of lipopolysaccharide (LPS) and 2 μ g/ml of Concanavalin A (Sigma), respectively. More than 85% of cells were B

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or T lymphocytes as determined by FACS analysis. BM cells were cultured in medium containing 20% (vol./vol.) of L929 cell supernatant. More than 95% of cells harvested after 10-11 days were macrophages as determined by morphological analysis.

11.1.3. ADENOSINE DEAMINASE ASSAY

Isozyme-specific activity was detected in cell lysates by nondenaturing isoelectric focusing (IEF) (Multiphor II, Electrophoresis system, Pharmacia LKB, Piscataway, NJ) as described (Wilson et al., supra). Total protein concentration was determined for each sample using the Bio-Rad protein assay (Bio-Rad, Melville, NY). Fixed amounts of total protein were loaded on the IEF gels (300 μ g for peripheral blood cells (PBC), 150 μ g for BM, 120 μ g for spleen, macrophages and B lymphocytes, 75 μ g for T lymphocytes). After 12 minutes of staining reaction, the gels were fixed and the colorimetric intensity of each band was quantified using a computerized densitometer (Computing Densitometer, Molecular Dynamics, Sunnyvale, CA).

11.2. RESULTS

11.2.1. GENERATION OF RECOMBINANT RETROVIRUSES ENCODING HUMAN ADENOSINE DEAMINASE

Insertion of the human ADA sequences into the MFG retroviral vector was performed so as to position the initiation ATG codon of the ADA cDNA at the position in the subgenomic viral transcript identical to that normally occupied by the viral envelope ATG. No selectable marker exists in the vector. Studies by Bowtell and co-workers (Botwel et al., 1987, Mol. Biol. Med. 4:229-250; Botwell et al., 1988, J. virol. 62:2464-2473) and Ostertag (Beck-Engeser et al., 1991, Hum. Gene Therapy 2:61-70; which demonstrated the

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transcriptional activity of the myeloproliferative virus (MPSV) LTR in vectors. We generated derivatives of MFG-ADA which possessed either the enhancer of MPSV (positioned in the 3' MoLV LTR) or both 5' and 3' MoMLV LTRs in place of the Mo-MLV LTRs. In addition, previous studies have suggested the potential novel properties of the Moloney Friend Virus enhancer sequences (Holland et al., 1987, Proc. Natl. Acad. Sci. USA 84:8662-8666; Bösze et al, 1988, EMBO 5: 1615-1623; Thiesen et al, 1988, J. Virol. 62:614-618). Accordingly, MFG-ADA derivatives were generated with Mo-MLV enhancer sequences replaced by analogous Friend enhancer sequences. We also generated derivatives of MFG-ADA and the MPSV LTR containing derivative of MFG-ADA MPSV enhancer sequences which carry a mutation in the viral tRNA primary binding site, designated B2 (Barklis et al., 1986, Cell 47:391-397; Weiher et al., 1987, J. Virol. 61:2742-2746). Lastly, to provide a comparison of LTR-based vectors and vectors which employ internal promoters for expression of inserted genes, we generated α SGC-ADA. This vector utilizes a hybrid transcriptional element comprised of the human α globin promoter and CMV enhancer sequences and carries a deletion of enhancer sequences in the 3'LTR. The precise structure of each of the above constructs is described in Section 11.1.1., *supra*.

All of the above vectors were packaged in the Ψ CRE packaging cell line. For each vector, approximately 25 cloned packaging cell lines were tested for virus production, and the specific cell line that transmitted the correct proviral structure at the highest copy number was selected by Southern blot analysis for future use. To select virus-producer cell clones for the Mo-LTR/B2 and MPSV-Enh/B2 constructs, which have retained the B2 mutation and

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are capable of transmitting it to NIH 3T3 cells, we amplified by PCR and sequenced a 400 bp fragment encompassing the B2 mutation in both producer and infected cells. The mutation was represented in half
5 to one fourth of the virus-producer cell clones and in those cases in which it was present in producer cells, it was shown to be transmitted to target cells. After PCR analysis of 5 to 7 clones for each of the 2 constructs, high-titer clones were identified.

10 A Southern blot of DNA isolated from NIH 3T3 cells infected with virus obtained from selected producer clones representing each vector is shown in Figure 11C. The data indicate that the correct proviral structures are in all cases transmitted to
15 cells at high efficiency. The transmission efficiency of all viruses for transduction of NIH 3T3 cells was in a range of 0.7 to 3.6 copies of provirus per cell. These virus-producing clones were used to perform the experiments described in Sections 11.2.2. and 11.2.3.,
20 *infra*.

**11.2.2. DETECTION AND QUANTITATION OF HUMAN
ADENOSINE DEAMINASE ACTIVITY IN RECOMBINANT
MFG-ADA VECTOR-TRANSDUCED MURINE BONE MARROW
TRANSPLANT RECIPIENTS**

25 To investigate human adenosine deaminase (huADA) expression in cells derived from transduced hematopoietic stem cells *in vivo*, mice were transplanted with 2.5×10^5 to 4.5×10^6 BM cells that had been co-cultured with recombinant virus producing
30 cells as described in Section 11.1.2., *supra*. Fifteen to eighteen mice were transplanted per construct. A first step in assessing vector mediated gene expression involved the analysis of huADA enzyme activity in the peripheral blood cells (PBC) of
35 reconstituted animals 5-7 months after transplantation

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using the assay described in Section 11.1.3., supra (Figure 12). In this assay, human ADA activity can be readily separated from the murine ADA activity and the relative levels of the two activities can be estimated by densitometry measurements of the intensity of labeled in situ reaction products. This assay generates signals proportional to the amount of enzyme activity.

In mice repopulated with cells transduced with the recombinant MFG-ADA vectors, 90 out of 93 transplanted animals expressed huADA. In 83% of those transplanted animals (77/93), the level of expression of huADA was equal to or greater than the level of endogenous murine ADA (mADA) expression. In 14% of these mice (13/93), the levels of huADA were in the order of 1/4 to 3/4 of the murine ADA. Only 3% of, the animals (3/93) did not express the huADA at a detectable level. In mice reconstituted with the α G-SGC-infected cells, only 4 mice out of 11 expressed huADA at levels close to the the murine enzyme and 6 at much lower levels (Figure 12). The small percentage of mice which express huADA from this vector is probably due to the low copy number of provirus detected in the tissues of these animals. Based on the average ratio of human to murine activity observed from mouse to mouse in these studies, the data suggest that the MFG-ADA derivative which utilizes the MPSV LTRs rather than Mo-MLV LTRs yielded moderately more enzyme than the other constructs (about 2 fold). However, this data does not take into account potential differences in proviral DNA copy number.

11.2.3. LONG-TERM IN VIVO EXPRESSION OF HUMAN ADENOSINE-DEAMINASE BY VARIOUS HEMATOPOIETIC

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CELL LINEAGES FOLLOWING BONE MARROW TRANSPLANTATION

Based on the high frequency of bone marrow transplant recipients which demonstrated significant gene expression in the peripheral blood seven months after bone marrow transplantation, a smaller number of animals were further examined for vector expression in a number of different hematopoietic cell types at much longer times after transplantation (greater than one year). A first step in these studies was to reanalyze the mice previously analyzed for enzyme expression in peripheral blood. As is shown in Figure 12B, comparison of the relative amounts of human and mouse enzymes at the two time points (shown below each IEF gel track) indicates that little if any significant decrease in huADA expression occurred over time. As shown in the boxes labeled with the different vectors, approximately 80% of the expression observed at 7 months persists after one year. In mice engrafted with α SGC-ADA treated cells, a slightly more significant decrease in expression was observed.

In the next series of experiments, expression of huADA in different hematopoietic cell types was examined by fractionating each cell population and quantitating enzyme activity and proviral copy number. This analysis included 3-5 animals engrafted with cells transduced by either (i) MFG-ADA; (ii) MFG-ADA (+B2); (iii) MFG-ADA(Friend enhancer); (iv) MFG-ADA (MPSV-LTRs); or, (v) α -SGC-ADA. The cell populations subjected to enzyme and DNA analysis included whole bone marrow, whole spleen, and fractionated macrophages, T lymphocytes, and B lymphocytes. A compilation of all the data obtained, expressed in several different ways, is shown in Figure 13. Panel A displays data representing individual mice, while other panels represent average values.

Collectively, the data may be summarized as follows:

1. Average proviral copy number achieved by different viruses:

5 Figure 13, panel C, shows that a significant proportion of the different hematopoietic cell populations carry proviral sequences even at over one year post-transplantation. With the exception of the α -SGC-ADA animals, where fewer cells appear to carry provirus, each of the other vectors yielded comparable
10 proviral copy numbers (0.2-0.7 copies/cell) in all lineages. The error bars illustrate that there is, however, significant variation in proviral copy number from mouse to mouse. It is possible that specific
15 transduced stem cell clones contribute unequally to different lineages, causing the observed variations in copy number.

2. Average expression levels of huADA achieved by different viruses:

20 The results presented in Figure 13, panel D, indicate that on average there is a remarkably similar level of gene expression attained in the different hematopoietic cell lineages by the different vectors. This conclusion is quite important, for it suggests
25 that the vectors examined do not, to any great extent, exhibit tissue specificity of gene expression, and therefore may well be useful for a variety of applications in which expression is required in a specific cell lineage, yet permissible in other cells.

30 The results presented in Figure 13, panels B and D, demonstrate that while all vectors yield gene expression in different hematopoietic cell lineages, both the MoLTR-B2 and the MPSV-LTR derivatives of MFG-ADA appear to yield greater levels of gene expression
35 in most lineages in relation to the parental MFG-ADA

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vector. Because of the significant variation in expression levels from mouse to mouse, and the relatively small number of animals examined, it was important to provide statistical analysis of the data.

5 As shown in panels B and D of Figure 13, the bars marked with an asterisk indicate values that are statistically significant relative to values for the parental MFG-ADA vector. This data indicates that only the B2 and MPSV LTR derivatives show significant
10 differences in expression in relation to the MFG-ADA vector. In the case of the B2 vector, significance could not be established for the T lymphocyte lineage, due to the wide variation in expression levels observed from mouse to mouse. Similarly, statistical
15 significance could not be established for the macrophage lineage in the case of MPSV-LTR derivative of MFG-ADA.

Another useful way to express the data provided in Figure 13, panel A, is to consider the levels of
20 expression in each lineage per mg protein, rather than per proviral copy number per mg protein. This representation of the data is perhaps most relevant to an assessment of the overall performance of each vector, since it takes into account both the inherent
25 expression potential of the vector and the ability of the vector to transduce cells. Interestingly, because of the comparable proviral copy numbers achieved by each vector (except for the α SGC-ADA vector), the data in Figure 13, panel D, is quite similar to that
30 represented in panel B. Again, statistically significant differences in gene expression relative to MFG-ADA mouse were observed with both the B2 and MPSV-LTR vectors. In contrast to the data provided in panel B, expression of the B2 vector in macrophages

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was not significantly different than that achieved by MFG-ADA.

**12. EXAMPLE: MFG VECTOR DERIVATIVES
WITH IMPROVED SAFETY FEATURES**

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The MFG-S vector is a derivative of the MGF vector that was designed to even further minimize the possibility of the formation of replication-competent virus through homologous recombination events.

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Specifically, MFG retains two intact overlapping open reading frames ("ORFs") that encode the amino terminal portion of both the cell surface and cytoplasmic gag-pol polyproteins. These ORFs provide a target region for recombination events with viral structural coding

15

sequences present in the packaging cell line which could lead to the formation of replication-competent virus. In order to minimize this already remote possibility, the MFG-S vector was constructed so that three specific mutations have been introduced into the viral gag region to disrupt the ORFs and thereby minimize any possibility of the expression of either cell surface or cytoplasmic gag-related polypeptides of any appreciable size.

20

The specific mutations to the MFG retroviral vector to produce the MFG-S retroviral vector are shown by direct comparison of the MFG and MFG-S DNA sequences in Figure 14. These are an A to T change at nucleotide 1256 and a C to T mutation at nucleotide 1478. These mutations create stop codons downstream from the initiation codons for the cell surface or cytoplasmic gag polypeptides and reduce the corresponding gag-related ORFs to 84 and 15 nucleotides, respectively. A third mutation was engineered into the DNA sequence which changes a T to an A at nucleotide 1273. This change does not effect the ORF but is a compensatory change that preserves

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the potential for base pairing with nucleotide 1252 preserving a stem loop that is theoretically important for the packaging function.

The salient features of the MFG-S retrovirus and their location by nucleotide position are listed in Table II below.

TABLE II

	FEATURE	NUCLEOTIDE POSITION
10	5' - murine flanking sequences	1-396
	U3 region of 5' - LTR	397-845
	RNA cap site	846
	R region of 5' - LTR	846-913
	U5 region of 5' - LTR	914-990
15	Primer binding site	991-1007
	Splice donor site	1048-1052
	Start codons for cell surface gag ORF	1172-1174, 1202-1204
	Start codon for cytoplasmic gag ORF	1466-1468
20	MFG-S base pair substitutions	1256, 1273, 1478
	Splice acceptor site	1983-1991
	Nco I restriction site	2276-2281
	Bam HI restriction site	2285-2290
25	U3 region of 3' - LTR	2429-2877
	R region of 3' - LTR	2878-2945
	Polyadenylation signal	2923-2929
	U5 region of 3' - LTR	2946-3022
30	3' - mouse flanking sequences	3023-3718

Figure 15 shows the structural features of the MFG-S retroviral vector. The MFG-S vector consists of the following parts: (1) a Mo-MuLV DNA fragment containing the 5' LTR and downstream sequence extending to the Nar I site at nucleotide position 1039 (Nar I was

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converted to Nde I site); (2) a Sma I linker at nucleotide position 626 of the retroviral sequence; (3) a Mo-MuLV DNA fragment extending from the Nde I site at position 5401 to an Xba I site at nucleotide position 5674; (4) a synthetic double-stranded DNA fragment containing an Nco I site (CTAGACTTGCCATGGCGCGATC); (5) a Mo-MuLV fragment extending from the Cla I (converted to a Bam HI site) site at nucleotide position 7672 through the 3' LTR; and (6) pBR322 bacterial plasmid sequences. Nucleotide substitutions were made at position 1256, 1273, and 1478 of the Mo-MuLV sequence in the MFG-S vector. These nucleotide positions are relative to the Hind III site of the vector (see complete nucleotide sequence of MFG-S in Figure 17). The proviral transcription unit of MFG is flanked by 396 nucleotides of mouse genomic sequences on the 5' end and 697 nucleotides on the 3' end. cDNA sequences can be inserted between the unique Nco I and Bam HI sites. The flanking mouse genomic and proviral sequences are cloned between the Hind III and Eco RI sites of pBR322. The Bam HI site in pBR322 was eliminated.

Figure 16 provides a circular restriction map of the MFG-S vector. Figure 18 provides a restriction map of the MFG vector in table form.

13. BIOLOGICAL DEPOSITS

On October 3, 1991, Applicants have deposited with the American Type Culture Collection, Rockville, Md., USA (ATCC) the plasmid MFG with the factor VIII insertion, described herein ATCC accession no. 68726, plasmid MFG with the tPA insertion, described herein, given ATCC accession no. 68727, the plasmid α -SGC, described herein, with the factor VIII insertion, given ATCC accession no. 68728, and plasmid α -SGC with

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the tPA insertion, described herein, given ATCC accession no. 68729. On October 9, 1991, Applicants have deposited with the American Type Culture Collection, Rockville, MD, USA (ATCC) the plasmid MFG, 5 described herein, given ATCC accession no. 68754, and plasmid α -SGC, described herein and given ATCC accession no. 68755. These deposits were made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of 10 Microorganisms for the purposes of patent procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture for 30 years from date of deposit. The organisms will be made available by ATCC under the terms of the Budapest 15 Treaty, and subject to an agreement between Applicants and ATCC which assures unrestricted availability upon issuance of the pertinent U.S. patent. Availability of the deposited strains is not to be construed as a license to practice the invention in contravention of 20 the rights granted under the authority of any government in accordance with its patent laws.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific 25 embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the following claims.

All patent, patent applications, and publications cited herein are hereby incorporated by reference.

30

35

WHAT IS CLAIMED IS:

1. A recombinant retroviral vector, said vector comprising in operable combination, a 5' LTR and a 3' LTR derived from a retrovirus of interest, and an insertion site for a gene of interest, wherein said vector does not contain a complete gag, env, or pol gene.
2. A recombinant retroviral vector according to Claim 1, said vector further comprising, a portion of a gag coding sequence.
3. A recombinant retroviral vector according to Claim 2, wherein said gag coding sequence comprises, a splice donor site and a splice acceptor site, wherein said splice acceptor site is located upstream from said gene of interest.
4. A recombinant retroviral vector according to Claim 3, said vector further comprising gag transcriptional promoter functionally positioned such that a transcript of a nucleotide sequence inserted into said insertion site is produced, wherein said transcript comprises gag 5' untranslated region.
5. A recombinant retroviral vector according to Claim 4, wherein said vector does not contain a selectable marker.
6. A recombinant retroviral vector according to Claim 5, wherein said vector is MFG having the identifying characteristics of ATCC 68,754.

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7. A recombinant retroviral vector according to Claim 1, said vector further comprising a gene for expression inserted into said insertion site.

5 8. A recombinant retroviral vector according to Claim 7, wherein said gene for expression is selected from the group consisting of: a hormone, an enzyme, a receptor, and a drug.

10 9. A recombinant retroviral vector according to Claim 8, wherein said gene for expression is factor VIII or tPA.

10. A recombinant retroviral vector according to
15 Claim 1, said vector further comprising, an alpha globin transcriptional promoter.

11. A recombinant retroviral vector according to Claim 10, said vector further comprising, a portion
20 of the 5' untranslated region of the α -globin gene that is naturally joined to said alpha-globin transcriptional promoter.

12. A recombinant retroviral vector according to
25 Claim 11, said vector further comprising, an enhancer sequence, wherein said enhancer is not in said 5' or 3' LTR.

13. A recombinant retroviral vector according to
30 Claim 12, wherein an enhancer sequence is located upstream from said transcriptional promoter.

14. A recombinant retroviral vector according to Claim 13, wherein said enhancer sequence is a
35 cytomegalovirus enhancer sequence.

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15. A recombinant retroviral vector according to Claim 14, wherein said vector is α -SGC and having the identifying characteristics of ATCC No. 68755.

5 16. A recombinant retroviral vector according to Claim 10, wherein said 3' LTR does not contain a functional enhancer sequence.

10 17. A recombinant retroviral vector according to Claim 10, said vector further comprising, a gene for expression inserted into said insertion site.

15 18. A recombinant retroviral vector according to Claim 10, wherein said gene for expression is selected from the group consisting of a hormone, an enzyme, a receptor, and a drug.

20 19. A recombinant retroviral vector according to Claim 18, wherein said gene for expression is factor VIII or tPA.

25 20. A recombinant retroviral cell line wherein said cell line has been transfected with the coding sequence of a retroviral vector of any of Claims 1 to 19.

30 21. A recombinant retroviral vector comprising in operable combination, a 5' LTR and a 3' LTR derived from a murine leukemia virus, and an insertion site for a gene of interest, wherein said vector does not contain a complete gag, env, or pol gene.

35 22. The recombinant retroviral vector of Claim 21, further comprising an exogenous enhancer.

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23. The recombinant retroviral of Claim 22, wherein the exogenous enhancer is derived from a myeloproliferative sarcoma virus.

5 24. The recombinant retroviral vector of Claim 22, wherein the exogenous enhancer is derived from Molony Friend Virus.

25. The recombinant retroviral vector of Claim
10 21, 22, 23, or 24, further comprising a B2 mutation.

26. The recombinant retroviral vector of Claim 21, 22, 23, or 24, wherein the 3' LTR is replaced with a 3' LTR derived from a myeloproliferative sarcoma
15 virus.

27. The recombinant retroviral vector of Claim 26, further comprising a B2 mutation.

20 28. The recombinant retroviral vector of Claim 21, 22, 23, or 24, wherein the 5' LTR is replaced with a 5' LTR derived from a myeloproliferative sarcoma virus.

25 29. The recombinant retroviral vector of Claim 28, further comprising a B2 mutation.

30. The recombinant retroviral vector of Claim 21, 22, 23, or 24, wherein both the 5' LTR and the 3' LTR are respectively replaced with a 5' LTR and a 3' LTR derived from a myeloproliferative sarcoma virus.
30

31. The recombinant retroviral vector of Claim 30, further comprising a B2 mutation.
35

32. A method for genetically modifying a hematopoietic stem cell such that the cell expresses the product of a gene of interest, comprising:

- 5 (a) engineering a recombinant retroviral vector according to Claim 21 to contain the gene of interest at the insertion site; and
(b) transducing the hematopoietic stem cell with the recombinant retroviral vector.

10 33. A method of treating a hematologic disease characterized by a defective gene in a hematopoietic cell in a patient comprising:

- (a) isolating allogenic, HLA-identical bone marrow cells from a donor;
15 (b) transducing the donor bone marrow cells with a recombinant retroviral vector, according to Claim 21, engineered to contain a normal gene corresponding to the defective gene at the vector insertion site;
20 (c) culturing the transduced donor bone marrow cells;
(d) destroying the patient's immune system; and,
(e) administering approximately $2-6 \times 10^8$
25 transfused donor bone marrow cells per kilogram body weight to the patient via intravenous infusion following destruction of the patients immune system.

30 34. An improved synthetic vascular graft, the improvement comprising a lining of autologous endothelial cells genetically modified to produce human tissue-type plasminogen activator on the luminal surface of the graft, wherein said endothelial cells
35 have been modified prior to implantation of the graft

by transducing parental endothelial cells with a
recombinant retroviral vector, according to any of
Claims 1-19, engineered to contain the coding sequence
for human tissue-type plasminogen activator at the
5 vector insertion site.

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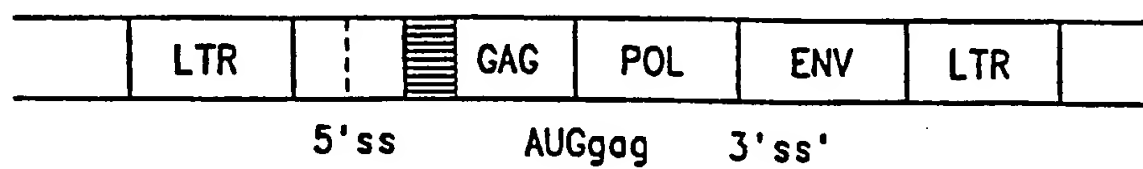
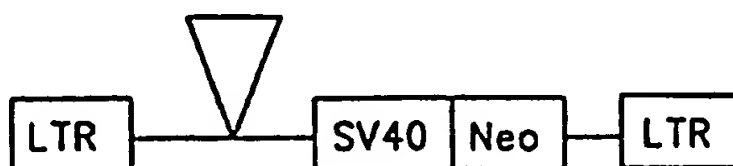
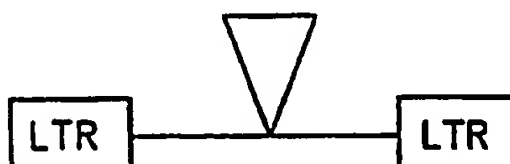
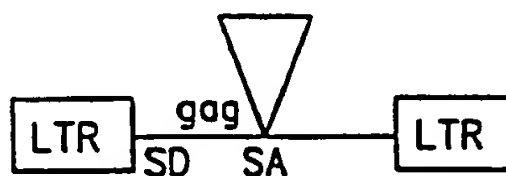
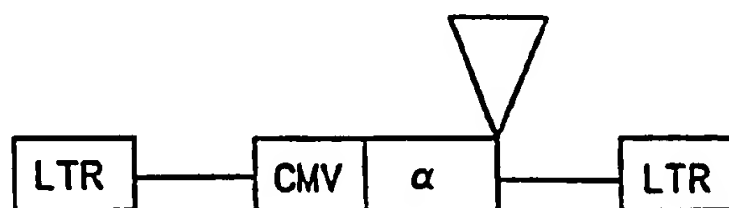


FIG.1

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FIG.2A
pLJFIG.2B
pEmFIG.2C
MFGFIG.2D
 α SGC

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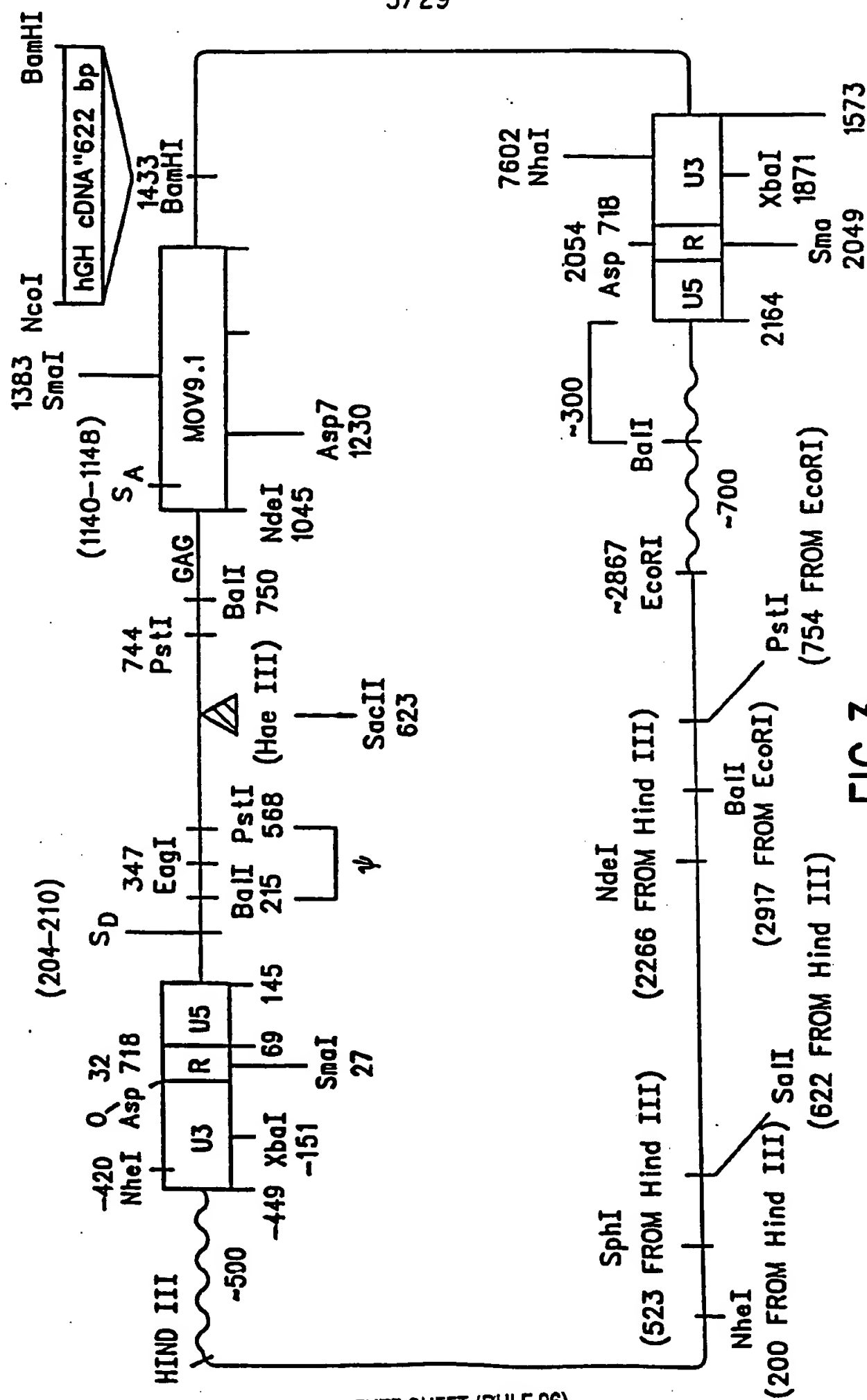


FIG. 3

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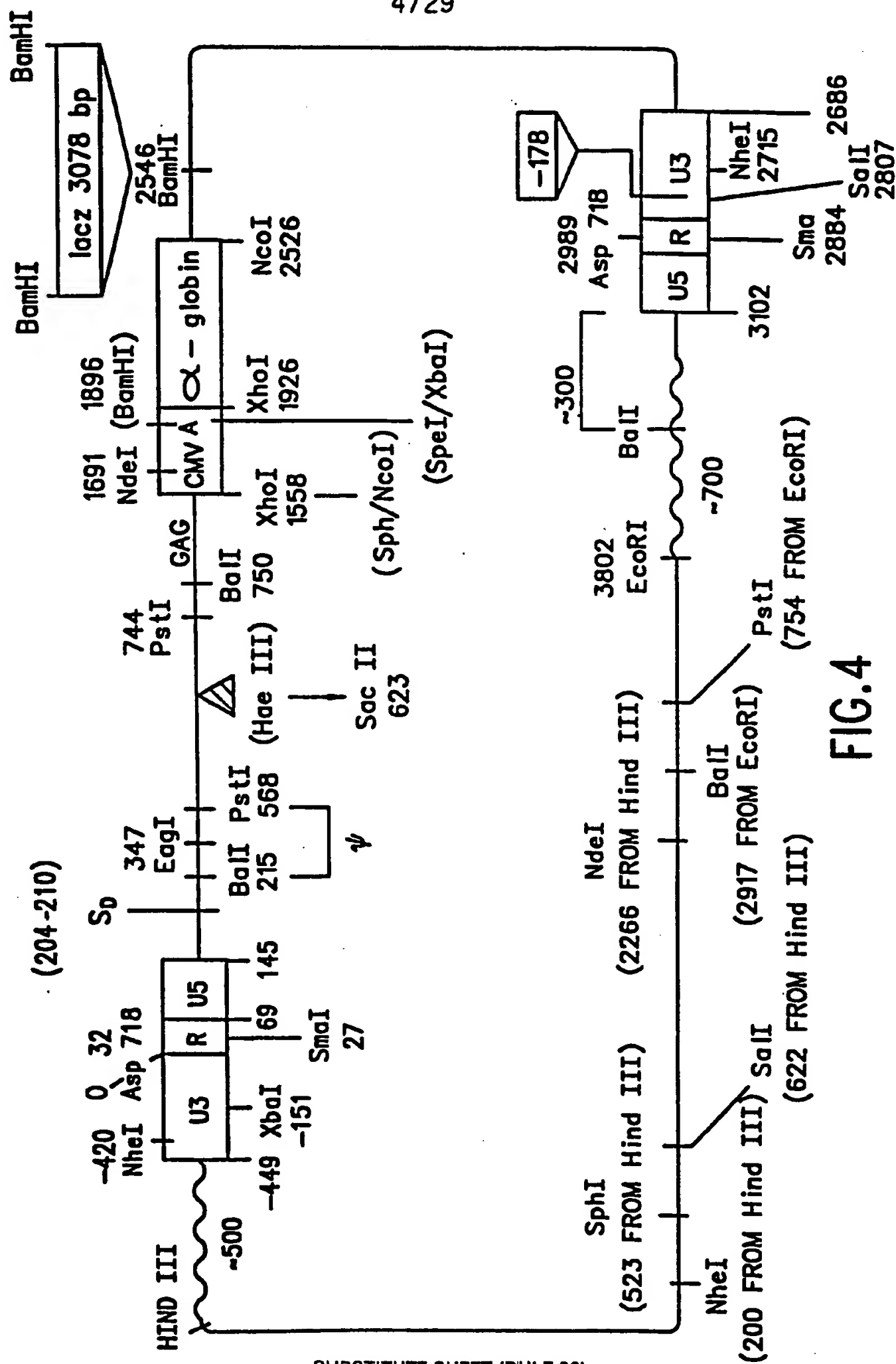


FIG. 4

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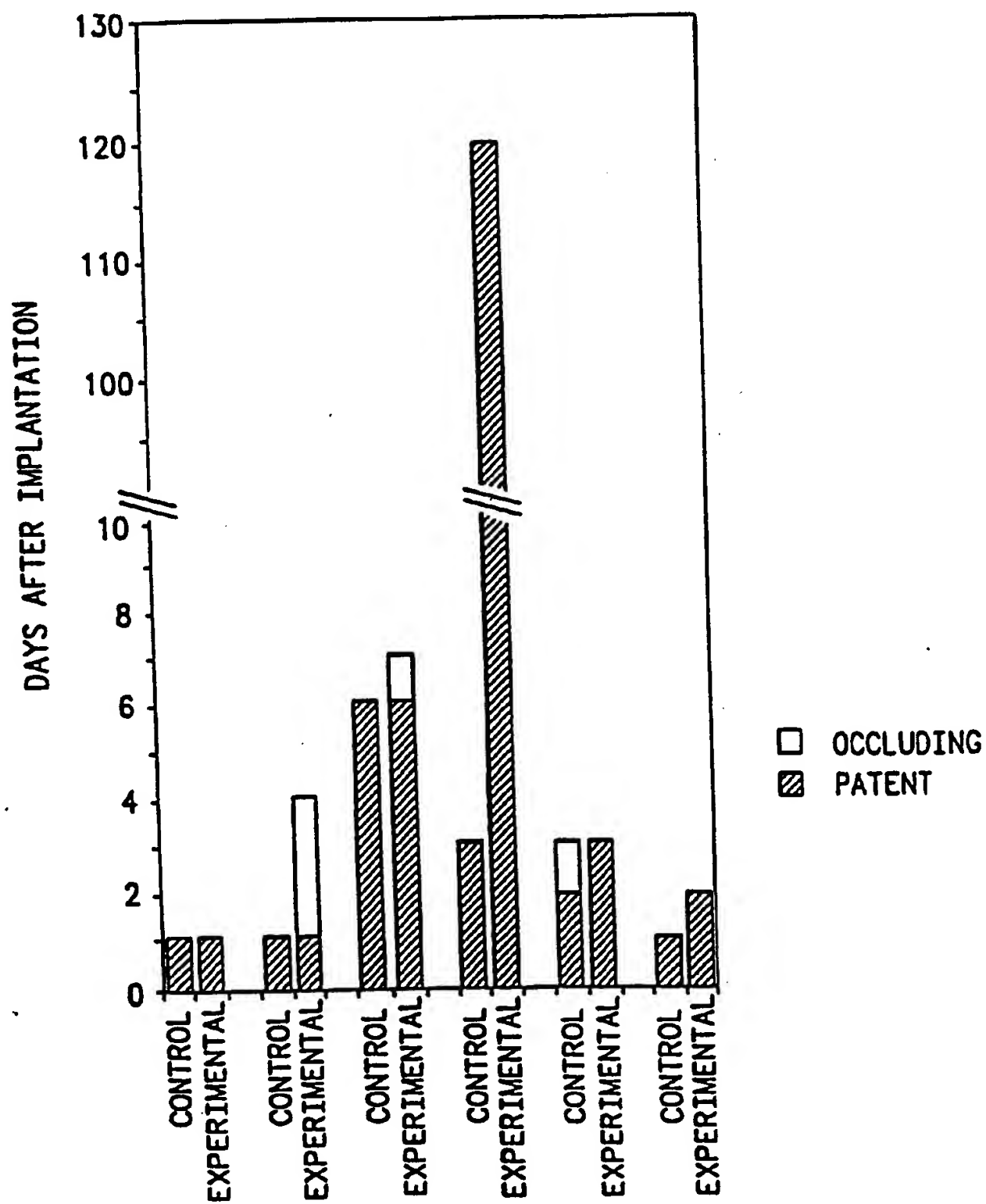


FIG.5

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FIG. 6A

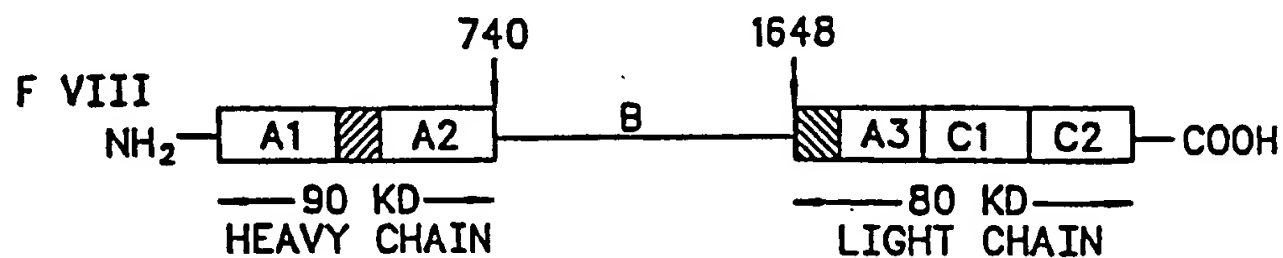


FIG. 6B

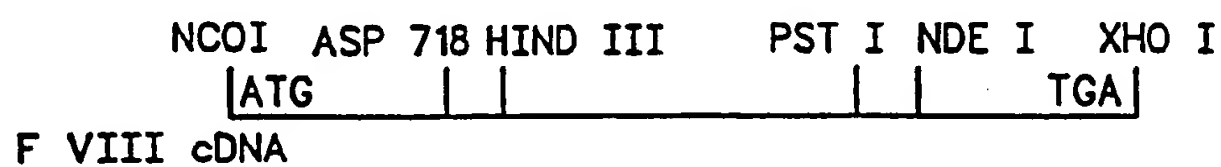
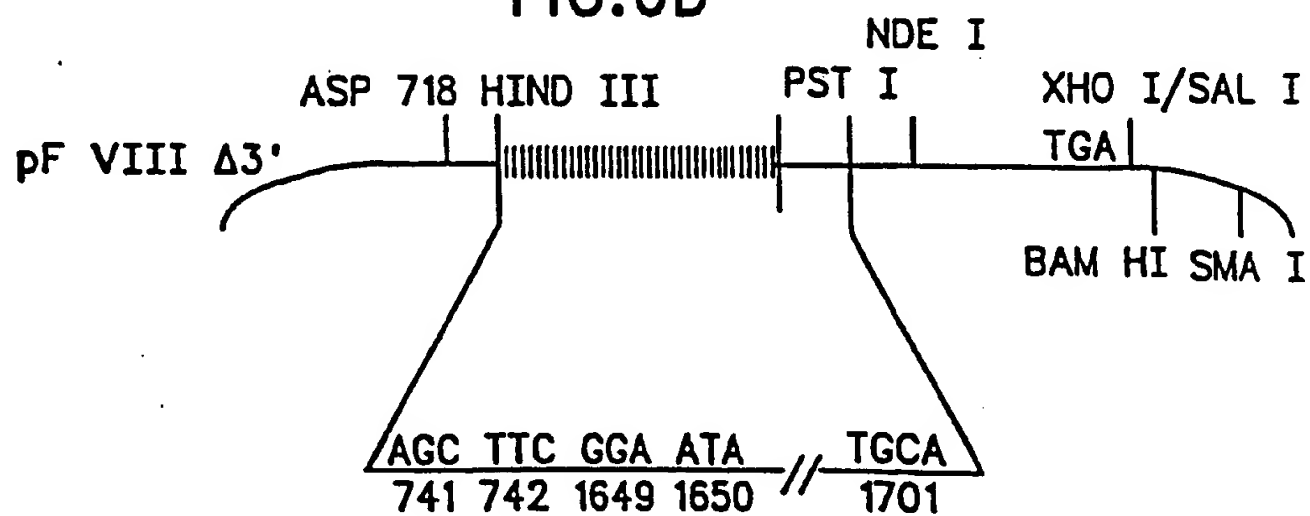


FIG. 6C



FIG. 6D



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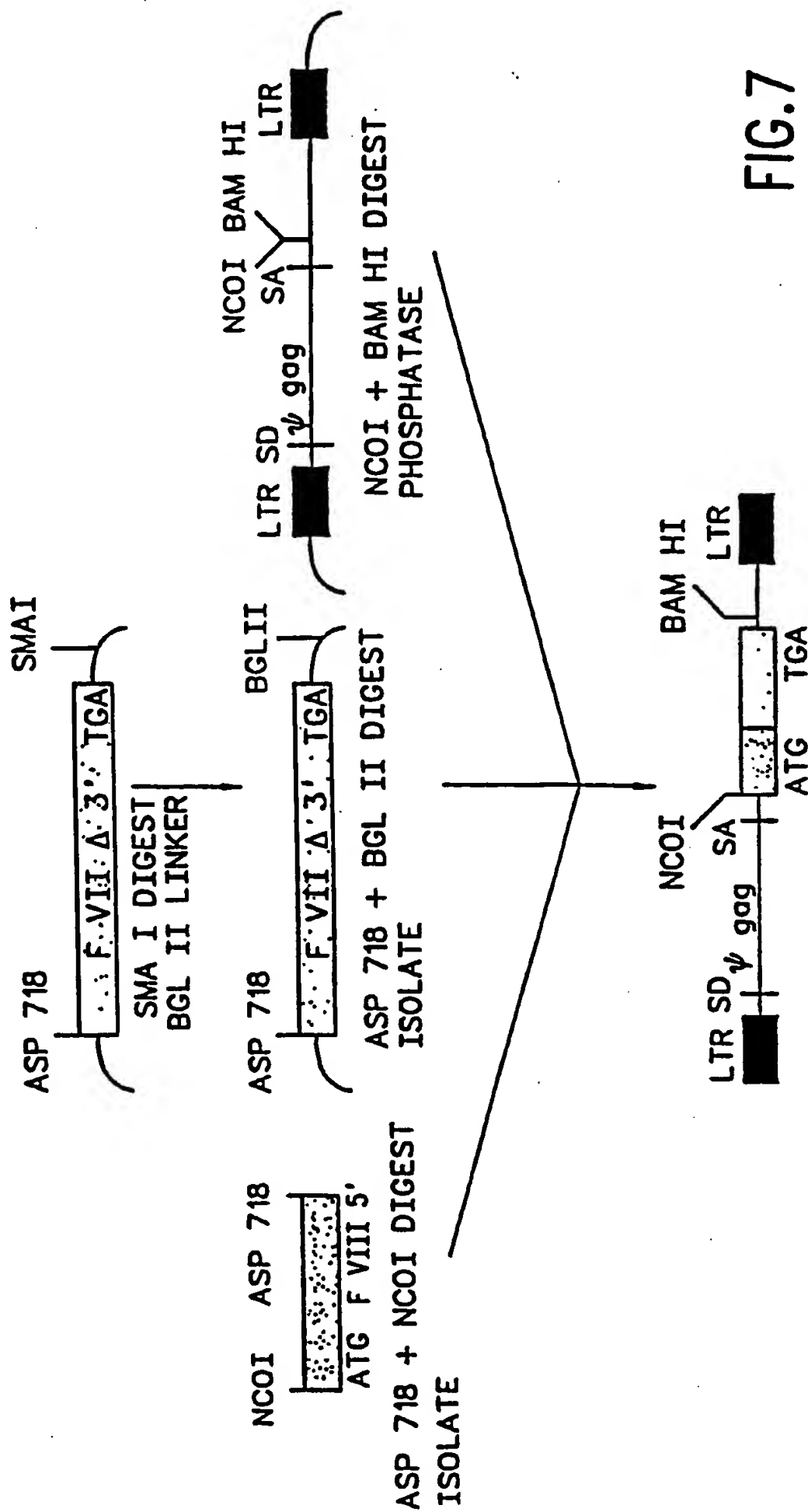


FIG. 7

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FIG.8

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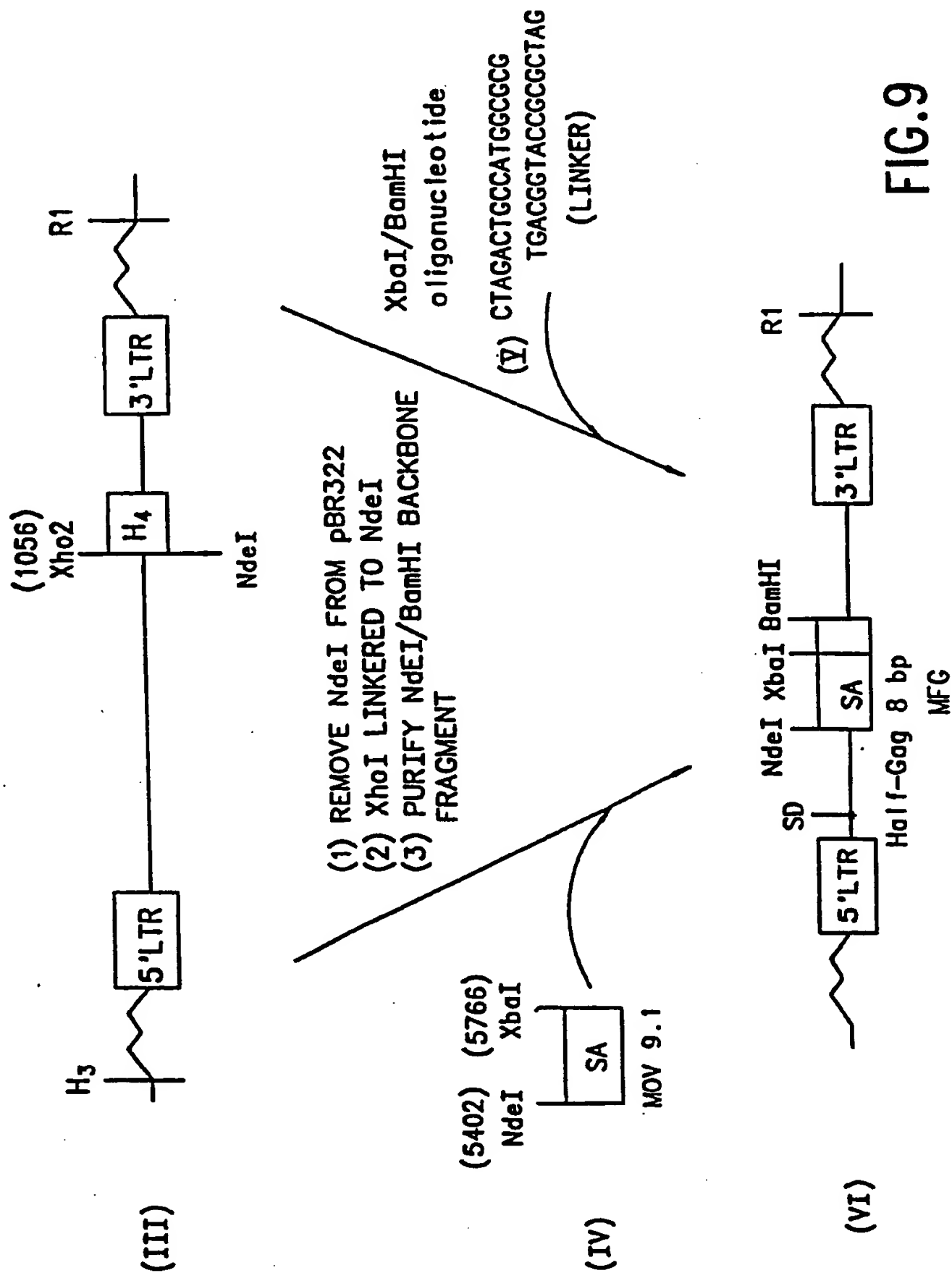
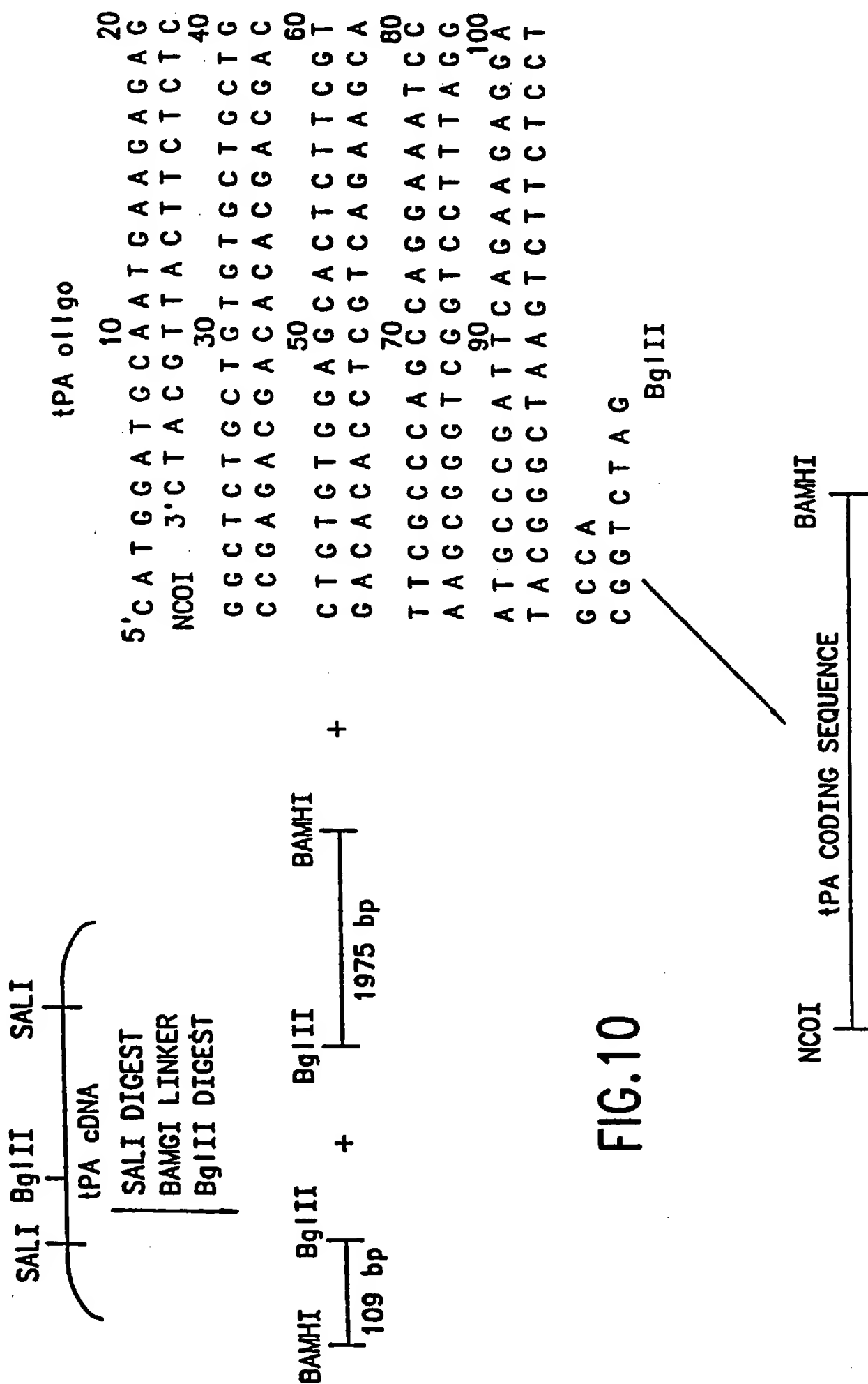
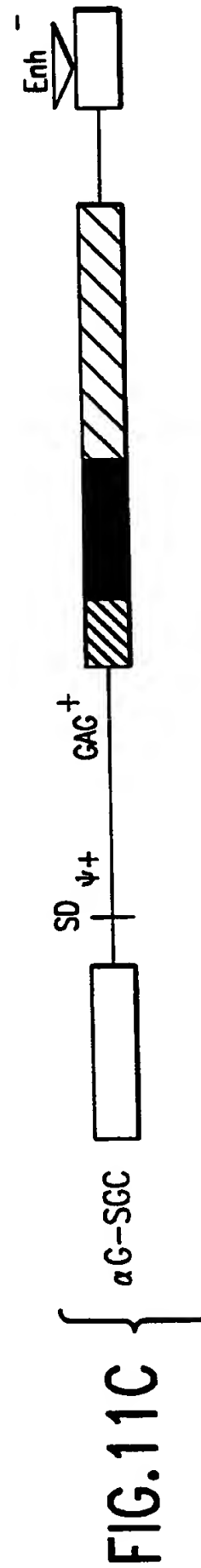
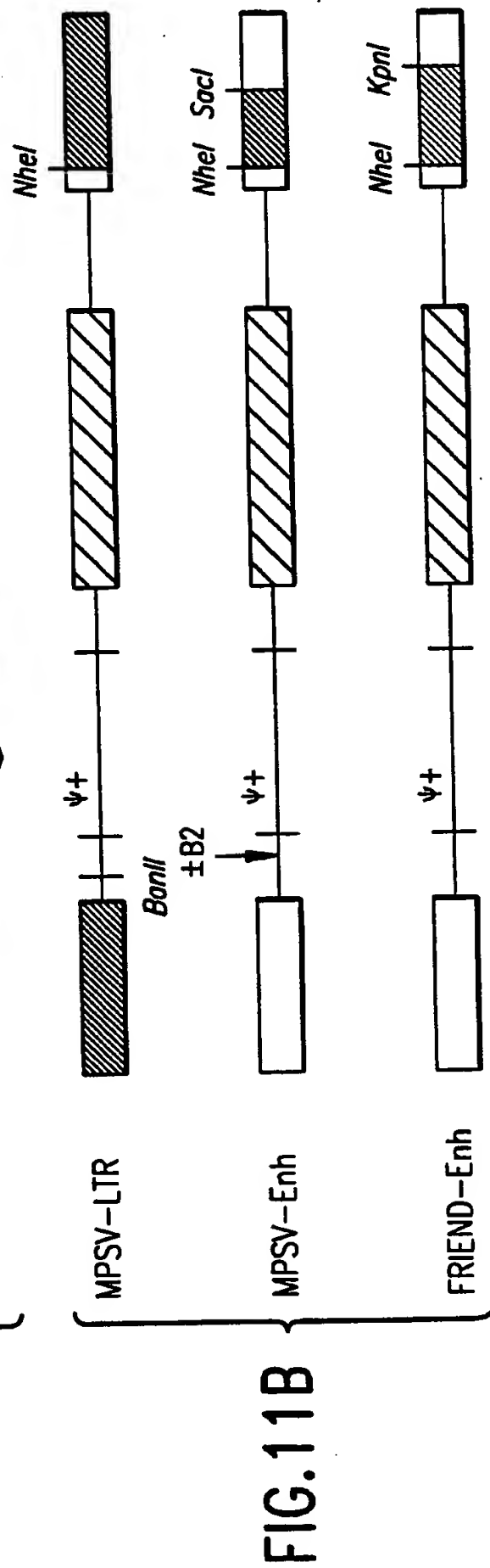
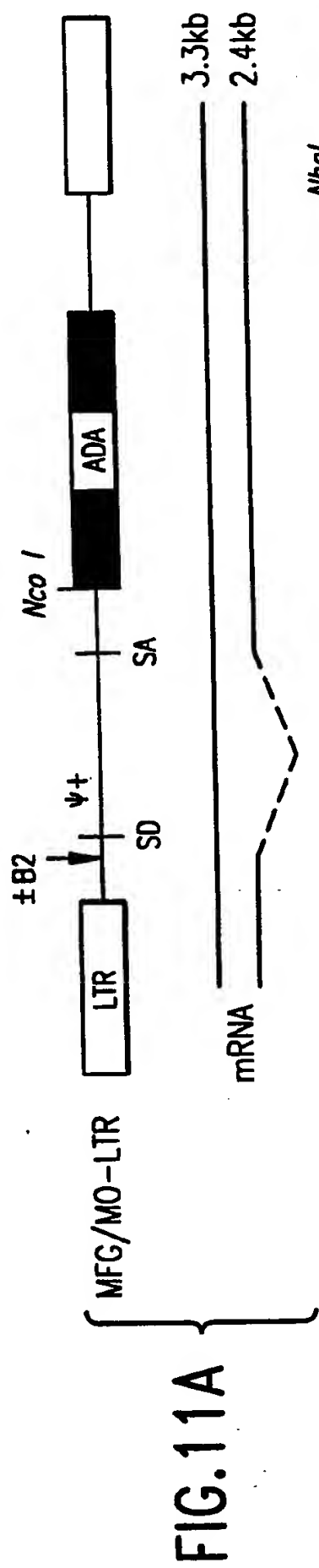


FIG.9

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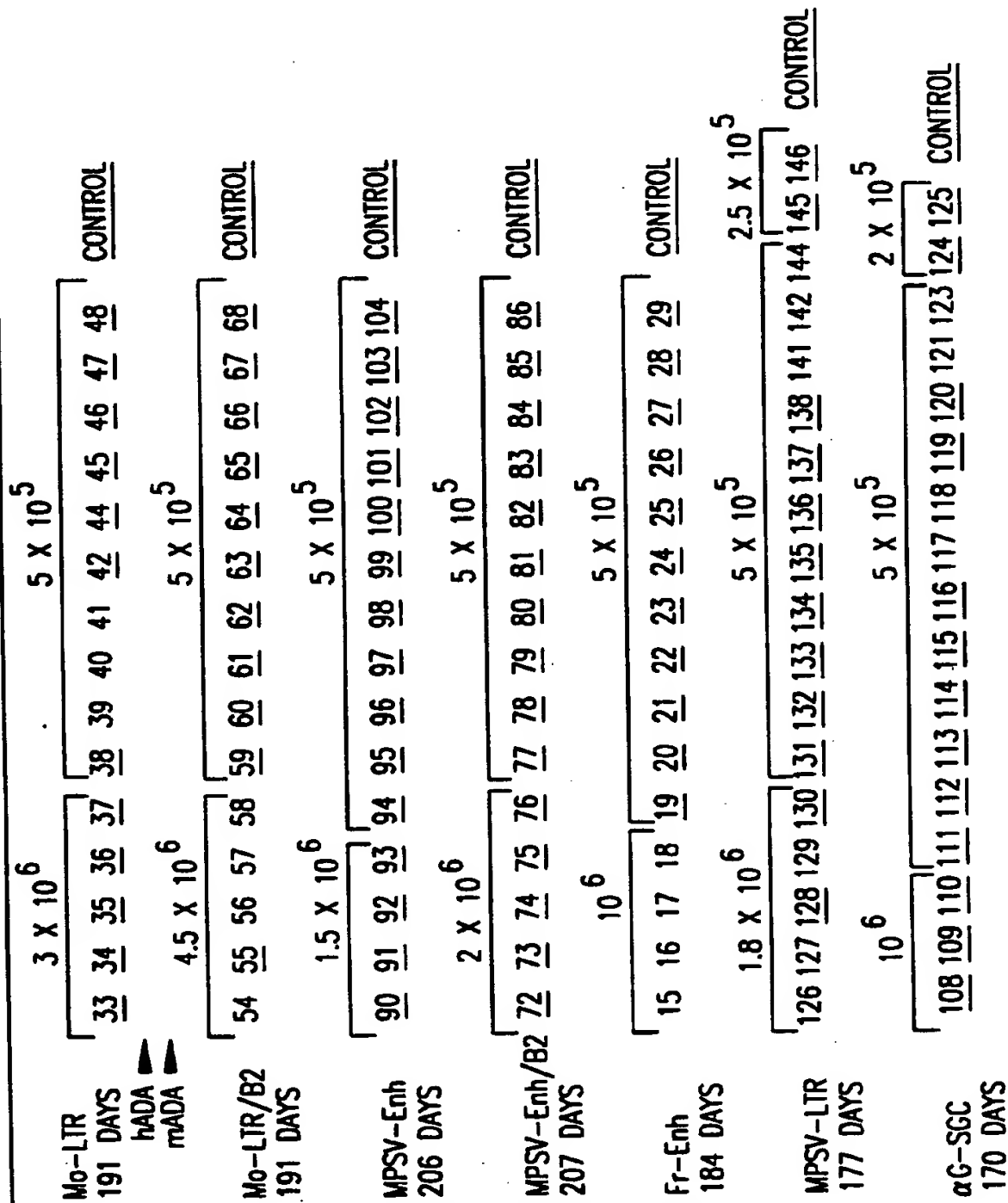
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	n_1 $r \geq 1$	n_2 $1 < r \leq 1/4$	$n_1 + n_2 / N$
Mo-LTR 191 DAYS hADA mADA	14	1	15/15
Mo-LTR/B2 191 DAYS	14	1	15/15
MPSV-Enh 206 DAYS	13	1	14/15
MPSV-Enh/B2 207 DAYS	9	6	15/15
Fr-Enh 184 DAYS	11	2	13/15
MPSV-LTR 177 DAYS	16	2	18/18
α G-SGC 170 DAYS	0	4	4/17
	77	17	94/110

FIG.12B



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	DAYS AFTER BMT											
	hADA						mADA					
	191		402		191		402		191		402	
Mo-LTR	#39	0.9	1.0	#40	1.4	0.7	#41	1.0	0.8	#58	1.7	1.2
80% (n=3)												
Mo-LTR/B2	#54	1.8	1.5	#56	1.8	1.1	#57	1.8	2	#58	1.7	1.2
82% (n=4)												
Fr-Enh	#15	1.6	1.3	#16	1.4	0.8	#17	1.2	1.6	#18	1.2	0.6
80% (n=4)												
MPSV-LTR	#126	2.2	1.7	#127	2.2	2.0	#129	1.8	ND	#141	ND	ND
77% (n=4)												
α G-SGC	#117	0.6	0.4	#121	0.05	0.03	#123	ND	ND	#118	0.8	0.4
59% (n=3)												
										</		

FIG. 12C

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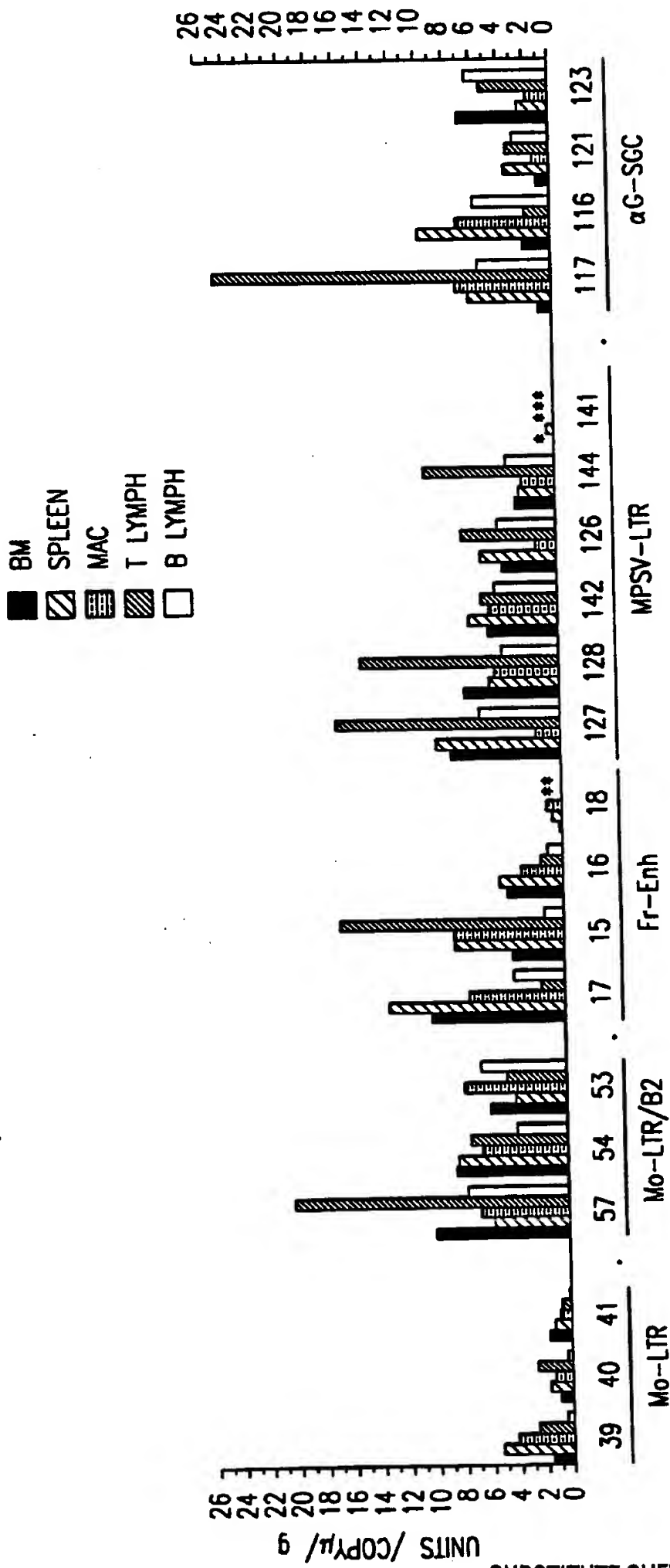


FIG.13A

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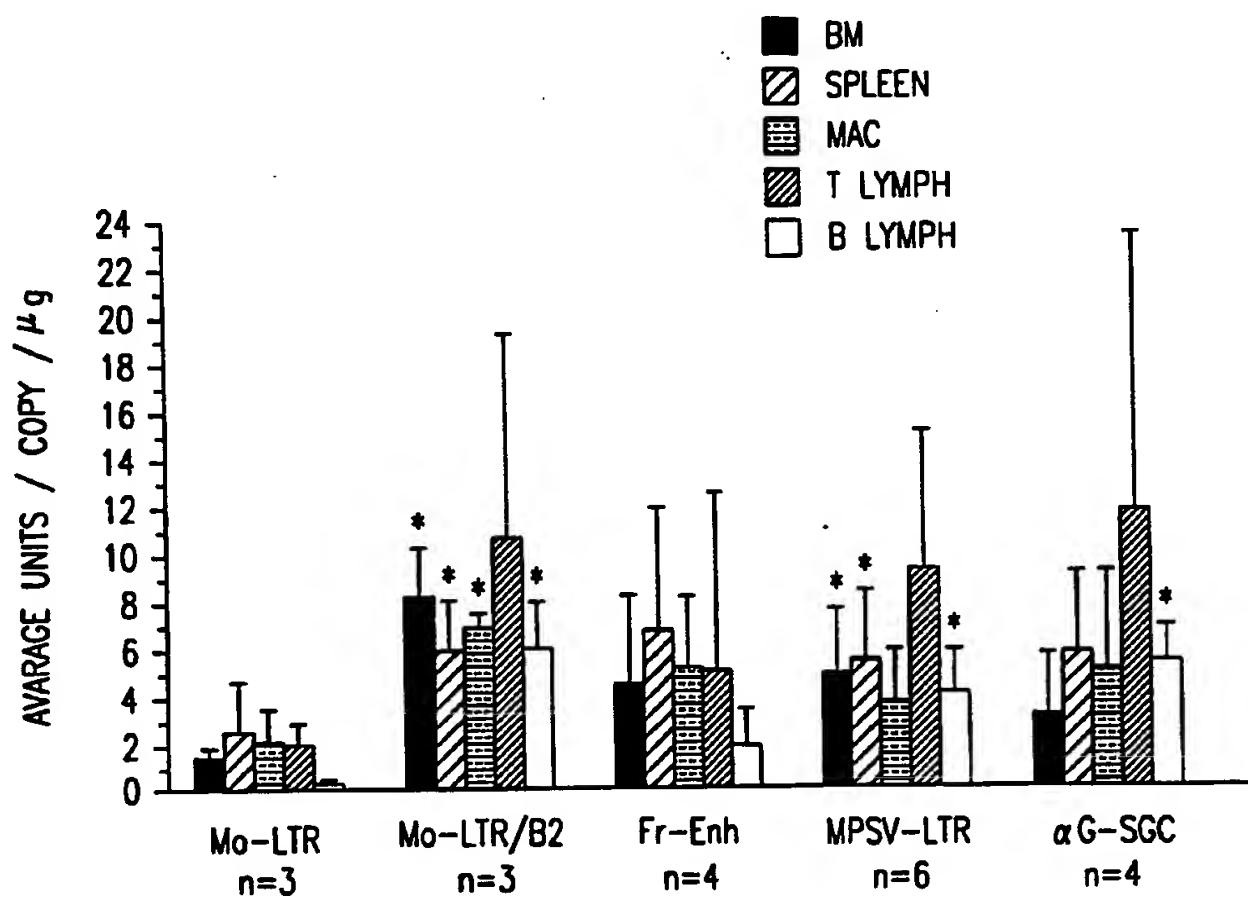


FIG. 13B

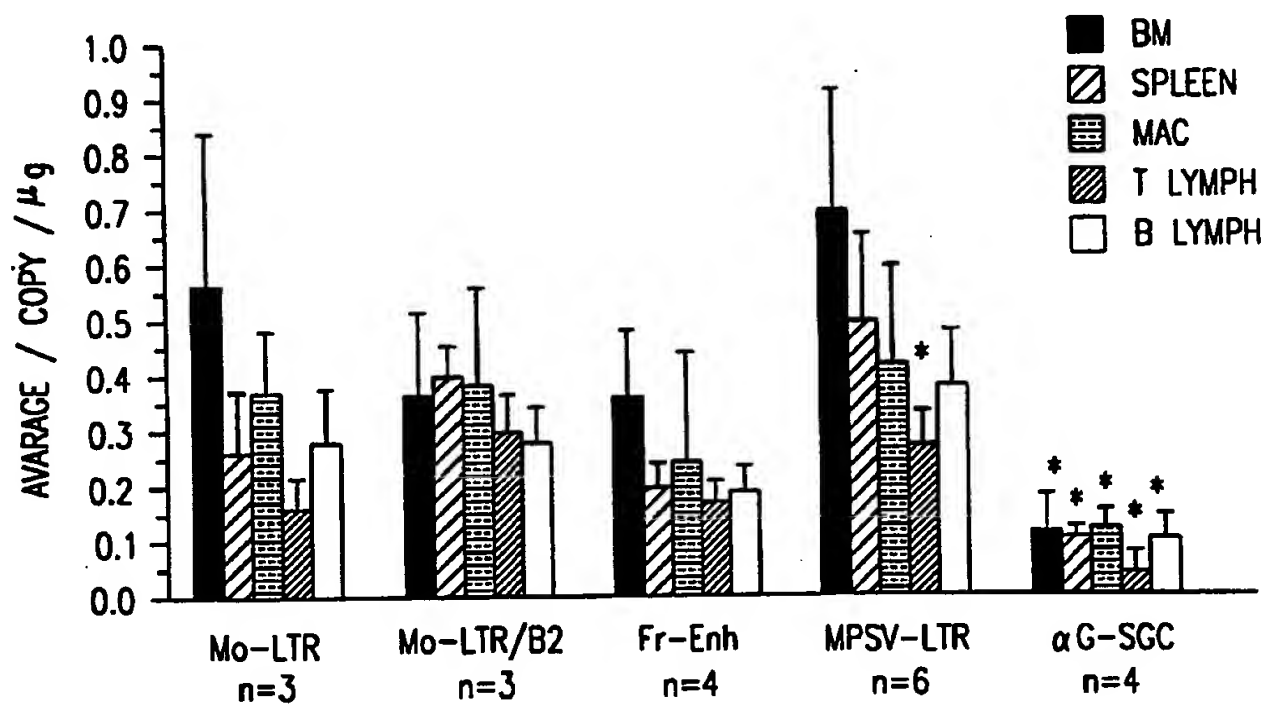


FIG. 13C

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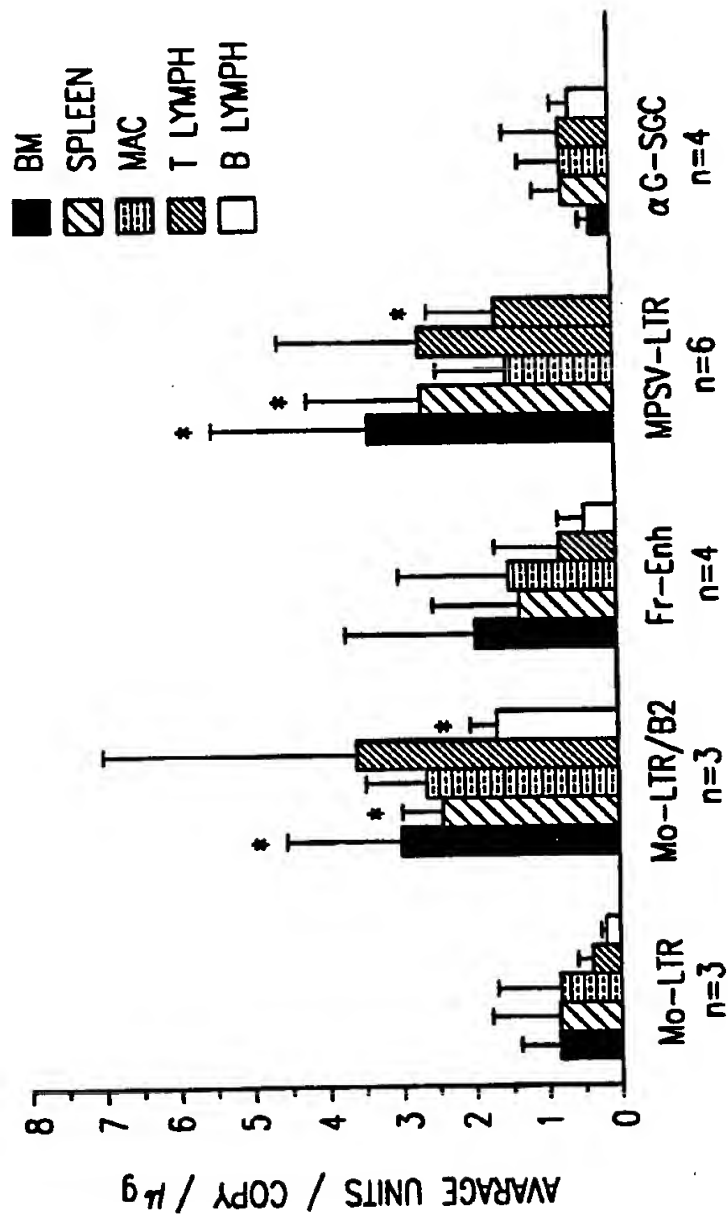


FIG.13D

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[illegible]

FIG. 14

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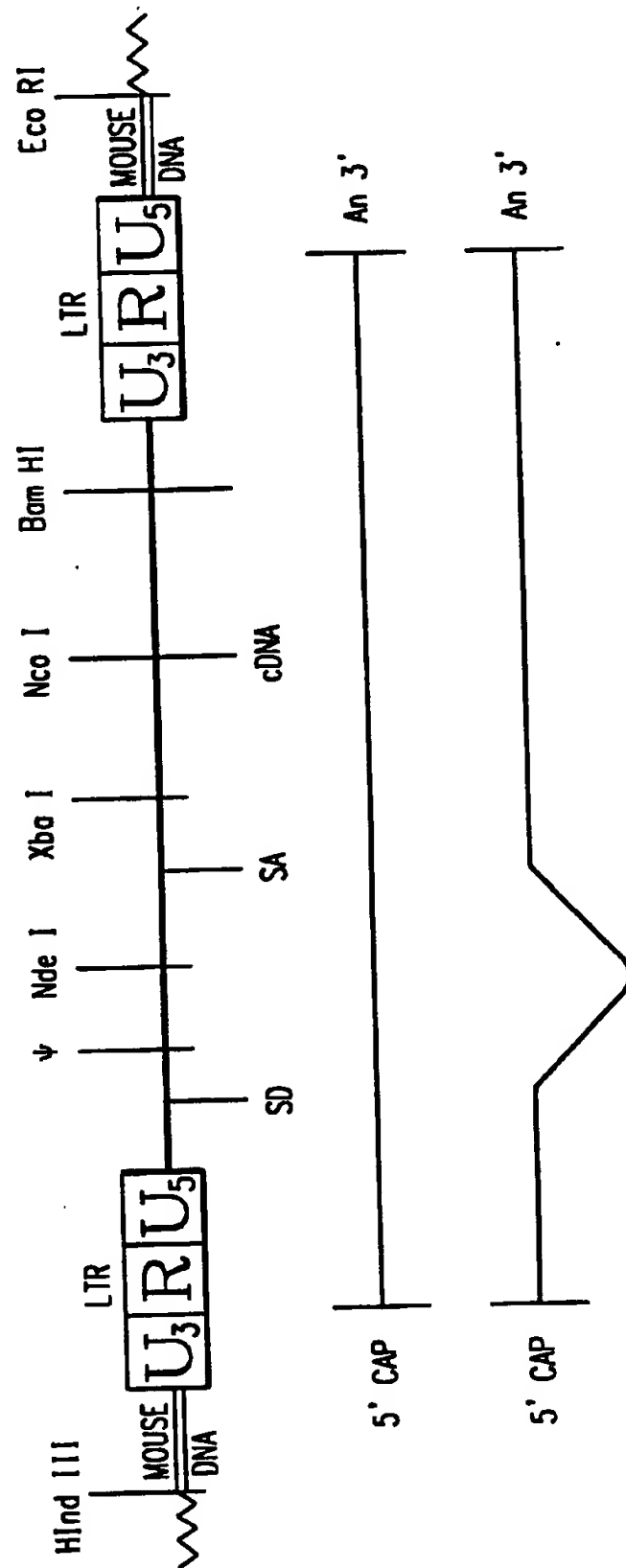


FIG.15

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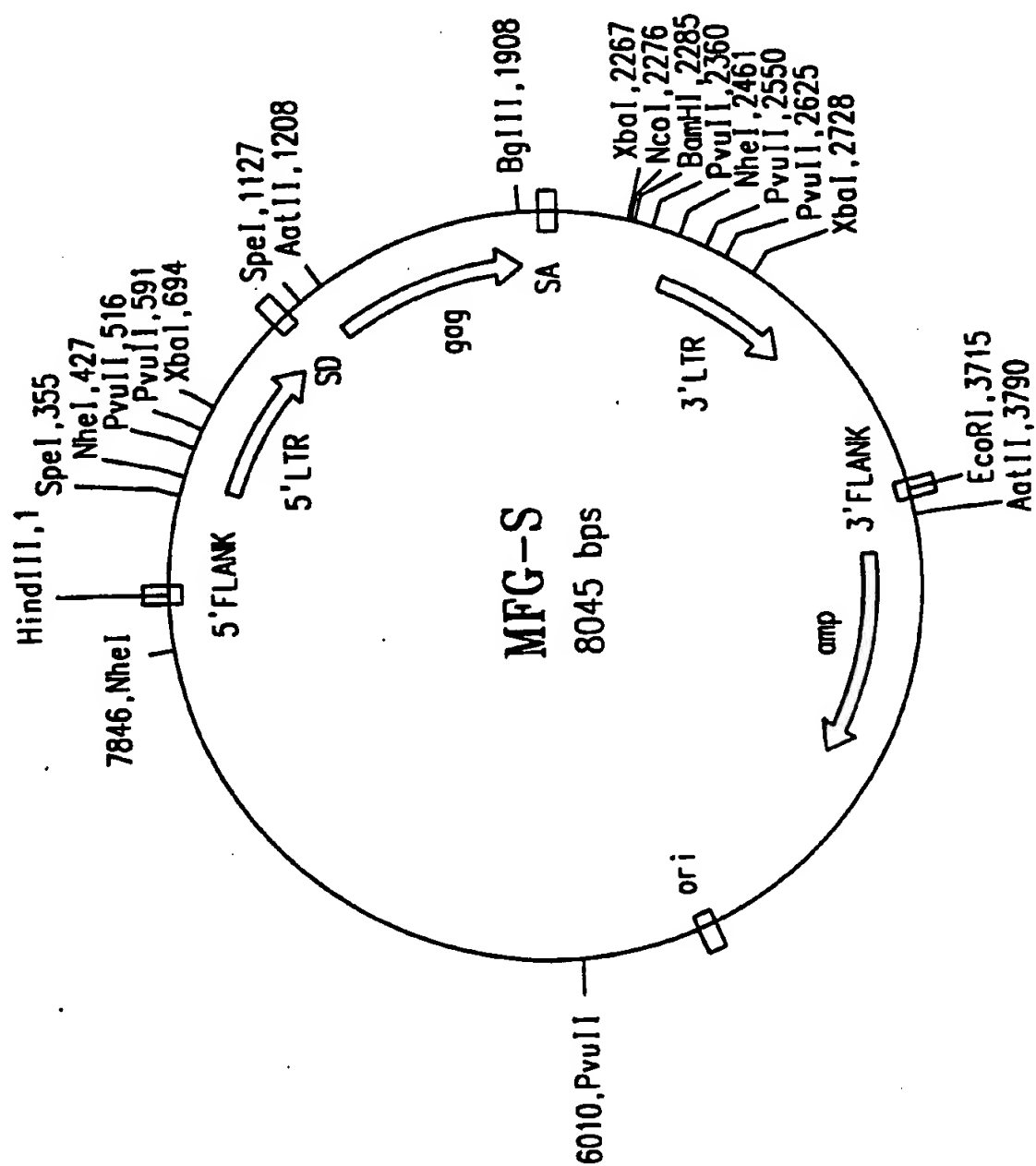


FIG.16

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1 AAGCTTTGCT CTTAGGAGTT TCCTAATACA TCCCAAAC TC AAATATATAA AGCATTGAC
TTCGAAACGA GAATCCTCAA AGGATTATGT AGGGTTTGAG TTTATATATT TCGTAAACTG

61 TTGTTCTATG CCCTAGGGGG CGGGGGGAAG CTAAGCCAGC TTTTTTTAAC ATTTAAAATG
AACAAGATAC GGGATCCCCC GCCCCCTTC GATTCCGTCG AAAAAAATTG TAAATTTTAC

121 TTAATTCCAT TTAAATGCA CAGATGTTTT TATTTCATAA GGGTTTCAAT GTGCATGAAT
AATTAAGTA AAATTTACGT GTCTACAAAA ATAAAGTATT CCCAAAGTTA CACGTACTTA

181 GCTGCAATAT TCCTGTTACC AAAGCTAGTA TAAATAAAAA TAGATAAACG TGGAAATTAC
CGACGTTATA AGGACAATGG TTTCGATCAT ATTTATTTTT ATCTATTTGC ACCTTTAATG

241 TTAGAGTTTC TGTCATTAAC GTTTCCTTCC TCAGTTGACA ACATAAATGC GCTGCTGAGC
AATCTCAAAG ACAGTAATTG CAAAGGAAGG AGTCAACTGT TGTATTTACG CGACGACTCG

301 AAGCCAGTTT GCATCTGTCA GGATCAATTT CCCATTATGC CAGTCATATT AATTACTAGT
TTCGGTCAAA CGTAGACAGT CCTAGTTAAA GGGTAATACG GTCAGTATAA TTAATGATCA

361 CAATTAGTTG ATTTTTATTT TTGACATATA CATGTGAATG AAAGACCCCA CCTGTAGGTT
GTTAATCAAC TAAAAATAAA AACTGTATAT GTACACTTAC TTTCTGGGGT GGACATCCAA

421 TGGCAAGCTA GCTTAAGTAA CGCCATTTTG CAAGGCATGG AAAAATACAT AACTGAGAAT
ACCGTTCGAT CGAATTCATT GCGGTAAAC GTTCCGTACC TTTTATGTA TTGACTCTTA

481 AGAAAAGTTC AGATCAAGGT CAGGAACAGA TGAACAGCT GAATATGGGC CAAACAGGAT
TCTTTTCAAG TCTAGTTCCA GTCCTGTCT ACCTTGTCGA CTTATACCCG GTTGTCTTA

541 ATCTGTGGTA AGCAGTTCTT GCGGCGGCTC AGGGCCAAGA ACAGATGGAA CAGCTGAATA
TAGACACCAT TCGTCAAGGA CGGGGCGAG TCCCGGTTCT TGTCTACCTT GTCGACTTAT

601 TGGGCCAAAC AGGATATCTG TGGTAAGCAG TTCCTGCCCC GGCTCAGGGC CAAGAACAGA
ACCGGTTTG TCCTATAGAC ACCATTCTG AAGGACGGGG CCGAGTCCCG GTTCTTGTCT

661 TGGTCCCCAG ATGCGGTCCA GCCCTCAGCA GTTTCTAGAG AACCATCAGA TGTTTCCAGG
ACCAGGGGTC TACGCCAGGT CGGGAGTCGT CAAAGATCTC TTGGTAGTCT ACAAAGGTCC

721 GTGCCCCAAG GACCTGAAAT GACCCTGTGC CTTATTTGAA CTAACCAATC AGTTCGCTTC
CACGGGGTTC CTGGACTTTA CTGGGACACG GAATAAATT GATTGGTTAG TCAAGCGAAG

781 TCGCTTCTGT TCGCGGCTT CTGCTCCCCG AGCTCAATAA AAGAGCCAC AACCCCTCAC
AGCGAAGACA AGCGCGGAA GACGAGGGG TCGAGTTATT TTCTCGGGT TGGGGAGTG

FIG.17A

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841 TCGGGGCGCC AGTCCTCCGA TTGACTGAGT CGCCCGGGTA CCCGTGTATC CAATAAACC
AGCCCCCGCG TCAGGAGGCT AACTGACTCA GCGGGCCCAT GGGCACATAG GTTATTGGG

901 TCTTGCAGTT GCATCCGACT TGTGGTCTCG CTGTTCCCTG GGAGGGTCTC CTCTGAGTGA
AGAACGTCAA CGTAGGCTGA ACACCAGAGC GACAAGGAAC CCTCCAGAG GAGACTCACT

961 TTGACTACCC GTCAGCGGGG GTCTTTCATT TGGGGGCTCG TCGGGATCG GGAGACCCCT
AACTGATGGG CAGTCGCCCC CAGAAAGTAA ACCCCGAGC AGGCCCTAGC CCTCTGGGA

1021 GCCCAGGGAC CACCGACCCA CCACCGGGAG GTAAGCTGGC CAGCAACTTA TCTGTGTCTG
CGGGTCCCTG GTGGCTGGGT GGTGGCCCTC CATTGACCG GTCGTTGAAT AGACACAGAC

1081 TCCGATTGTC TAGTGTCTAT GACTGATTTT ATGCGCCTGC GTCGGTACTA GTTAGCTAAC
AGGCTAACAG ATCACAGATA CTGACTAAAA TACCGGACG CAGCCATGAT CAATCGATTG

1141 TAGCTCTGTA TCTGGCGGAC CCGTGGTGA ACTGACGAGT TCGGAACACC CGGCCGCAAC
ATCGAGACAT AGACCGCCTG GGCACCACCT TGA CTGCTCA AGCCTTGTGG GCCGGCGTTG

1201 CCTGGGAGAC GTCCAGGGA CTTCGGGGGC CGTTTTGTG GCCCGACCTG AGTCCTAAAA
GGACCCTCTG CAGGGTCCCT GAAGCCCCG GCAAAAACAC CGGGCTGGAC TCAGGATTTT

1261 TCCCGATCGT TTAGGACTCT TTGGTGCACC CCCCTTAGAG GAGGGATATG TGGTTCTGGT
AGGGCTAGCA AATCCTGAGA AACCACGTGG GGGGAATCTC CTCCTATAC ACCAAGACCA

1321 AGGAGACGAG AACCTAAAC AGTTCGCCG TCCGTCTGAA TTTTGTCTT CGGTTTGGA
TCCTCTGCTC TTGATTTT TCAAGGGCGG AGGCAGACTT AAAAACGAAA GCCAAACCCT

1381 CCGAAGCOGC GCGCGCGTC TTGTCTGCTG CAGCATCGTT CTGTGTTGTC TCTGTCTGAC
GGCTTCGGCG CGCGCGCAG AACAGACGAC GTCGTAGCAA GACACAACAG AGACAGACTG

1441 TGTGTTTCTG TATTTGTCTG AAAATATGGG CCCGGGCTAG ACTGTTACCA CTCCTTAAG
ACACAAAGAC ATAAACAGAC TTTTATACCC GGGCCCGATC TGACAATGGT GAGGGAATTC

1501 TTTGACCTTA GGTCACCTGA AAGATGTGCA GCGGATCGCT CACAACCAGT CGGTAGATGT
AAACTGGAAT CCAGTGACCT TTCTACAGCT CGCCTAGCGA GTGTTGGTCA GCCATCTACA

1561 CAAGAAGAGA CGTTGGGTTA CCTTCTGCTC TGCAGAATGG CCAACCTTTA ACGTCGGATG
GTTCTTCTCT GCAACCCAAT GGAAGACGAG ACGTCTTACC GGTGGAAAT TGCAGCCTAC

FIG.17B

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1621 GCCGCGAGAC GGCACCTTTA ACCGAGACCT CATCACCCAG GTTAAGATCA AGGTCTTTTC
CGGCGCTCTG CCGTGGAAT TGGCTCTGGA GTAGTGGGTC CAATTCTAGT TCCAGAAAAG

1681 ACCTGGCCCG CATGGACACC CAGACCAGGT CCCCTACATC GTGACCTGGG AAGCCTTGGC
TGGACCGGGC GTACCTGTGG GTCTGGTCCA GGGGATGTAG CACTGGACCC TTCGGAACCG

1741 TTTTGACCCC CCTCCCTGGG TCAAGCCCTT TGTACACCCT AAGCCTCCGC CTCCTCTTC
AAACTGGGG GGAGGGACCC AGTTCGGGA ACATGTGGGA TTCGGAGCGG GAGGAGAAGG

1801 TCCATCCGCC CCGTCTCTCC CCCTTGAACC TCCTCGTTCC ACCCCGCCTC GATCCTCCCT
AGGTAGGCGG GGCAGAGAGG GGGAACCTGG AGGAGCAAGC TGGGGCGGAG CTAGGAGGGA

1861 TTATCCAGCC CTCCTCTCTT CTCTAGGCGC CCCCATATGG CCATATGAGA TCTTATATGG
AATAGGTCCG GAGTGAGGAA GAGATCCCGG GGGGTATACC GGTATACTCT AGAATATACC

1921 GGCACCCCGG CCCCTTGTA ACTTCCCTGA CCCTGACATG ACAAGAGTTA CTAACAGCCC
CCGTGGGGGC GGGGAACATT TGAAGGGACT GGGACTGTAC TGTCTCAAT GATTGTCCGG

1981 CTCTCTCAA GCTCACTTAC AGGCTCTCTA CTTAGTCCAG CACGAAGTCT GGAGACCTCT
GAGAGAGGTT CGAGTGAATG TCCGAGAGAT GAATCAGGTC GTGCTTCAGA CCTCTGGAGA

2041 GGCGGCAGCC TACCAAGAAC AACTGGACCG ACCGGTGGTA CCTCACCCTT ACCGAGTCGG
CCGCGTCGG ATGGTCTTG TTGACCTGGC TGGCCACCAT GGAGTGGGA TGGCTCAGCC

2101 CGACACAGTG TGGGTCCGCC GACACCAGAC TAAGAACCTA GAACCTCGCT GGAAAGGACC
GCTGTGTCAC ACCCAGGCGG CTGTGGTCTG ATTCTTGGAT CTTGGAGCGA CCTTTCCTGG

2161 TTACACAGTC CTGCTGACCA CCCCCACCGC CCTCAAAGTA GACGGCATCG CAGCTTGGAT
AATGTGTCAG GACGACTGGT GGGGGTGGCG GGAGTTTCAT CTGCCGTAGC GTCGAACCTA

2221 ACACGCCGCC CACGTGAAGG CTGCCGACCC CGGGGGTGGG CCATCCTCTA GACTGCCATG
TGTGCGGCGG GTGCACTTCC GACGGCTGGG GCCCCACCT GGTAGGAGAT CTGACGGTAC

2281 GCGCGGATCC GGATTAGTCC AATTGTGTTA AGACAGGATA TCAGTGGTCC AGGCTCTAGT
CGCGCCTAGG CCTAATCAGG TTAAACAATT TCTGTCCTAT AGTCACCAGG TCCGAGATCA

2341 TTTGACTCAA CAATATCACC AGCTGAAGCC TATAGAGTAC GAGCCATAGA TAAATAAAA
AAACTGAGTT GTTATAGTGG TCGACTTOGG ATATCTCATG CTCGGTATCT ATTTTATTTT

FIG.17C

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2401 GATTTTATTT AGTCTCCAGA AAAAGGGGGG AATGAAAGAC CCCACCTGTA GGTITGGCAA
CTAAATAAA TCAGAGGTCT TTTTCCCCC TTACTTTCTG GGTGGACAT CCAAACCGTT

2461 GCTAGCTTAA GTAACGCCAT TTTGCAAGGC ATGGAATAAT ACATAACTGA GAATAGAGAA
CGATCGAATT CATTGCGGTA AAACGTTCCG TACCTTTTA TGTATTGACT CTTATCTCTT

2521 GTTCAGATCA AGGTCAGGAA CAGATGGAAC AGCTGAATAT GGGCCAAACA GGATATCTGT
CAAGTCTAGT TCCAGTCCTT GTCTACCTTG TCGACTTATA CCCGGTTTGT CCTATAGACA

2581 GGTAAGCAGT TCCTGCCCCG GCTCAGGGCC AAGAACAGAT GGAACAGCTG AATATGGGCC
CCATTGCTCA AGGACGGGGC CGAGTCCCGG TTCTTGCTA CTTGTGCGAC TTATACCGG

2641 AAACAGGATA TCTGTGGTAA GCAGTTCCTG CCCC GGCTCA GGGCCAAGAA CAGATGGTCC
TTTGTCTAT AGACACCATT CGTCAAGGAC GGGGCCGAGT CCGGTTCTT GTCTACCAGG

2701 CCAGATGCGG TCCAGCCCTC AGCAGTTTCT AGAGAACCAT CAGATGTTTC CAGGGTGCCC
GGTCTACGCC AGGTGCGGAG TCGTCAAAGA TCTCTTGTA GTCTACAAAG GTCCACGGG

2761 CAAGGACCTG AAATGACCCT GTGCCTTATT TGAATAACC AATCAGTTG CTTCTCGCTT
GTTCTGGAC TTTACTGGGA CACGGAATAA ACTGATTGG TTAGTCAAGC GAAGAGCGAA

2821 CTGTTGCGC GCTTCTGCTC CCGAGCTCA ATAAAGAGC CCACAACCCC TACTCGGGG
GACAAGCGC CGAAGACGAG GGGCTCGAGT TATTTCTCG GGTGTTGGG AGTGAGCCC

2881 CGCCAGTCCT CCGATTGACT GAGTCGCCC GGTACCCGTG TATCCAATAA ACCCTCTGC
CGGTCAGGA GGCTAACTGA CTCAGCGGGC CCATGGGCAC ATAGGTTATT TGGGAGAAG

2941 AGTTGCATCC GACTTGTTG CTGCTGTTT CTTGGGAGG TCTCCTCTGA GTGATTGACT
TCAACGTAGG CTGAACACCA GAGCGACAAG GAACCCTCC AGAGGAGACT CACTAACTGA

3001 ACCCGTCAGC GGGGTCTTT CACACATGCA GCATGTATCA AAATTAATTT GGTITTTTTT
TGGCAGTCG CCCCAGAAA GTGTGTACG CTACATAGT TTAATTAAA CCAAAAAAA

3061 CTTAAGTATT TACATTAAAT GGCCATAGTA CTAAAGTTA CATTGGCTTC CTTGAAATAA
GAATTCATAA ATGTAATTTA CCGGTATCAT GAATTTCAAT GTAACCGAAG GAACTTTATT

3121 ACATGGAGTA TTCAGAATGT GTCATAAATA TTTCTAATTT TAAGATAGTA TCTCCATTGG
TGTACCTCAT AAGTCTTACA CAGTATTTAT AAAGATTAAA ATTCTATCAT AGAGGTAACC

3181 CTTTCTACTT TTTCTTTTAT TTTTTTTTGT CCTCTGTCTT CCATTTGTTG TTGTTGTGT
GAAAGATGAA AAAGAAAATA AAAAAAACA GGAGACAGAA GGTAACAAC AACAACAACA

FIG.17D

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3241 TTGTTTGT TTGTTGTTGGT TGGTTGGTTA ATTTTTTTTT AAAGATCCTA CACTATAGTT
AACAAACAAA CAAACAACCA ACCAACCAAT TAAAAAATAA TTTCTAGGAT GTGATATCAA

3301 CAAGCTAGAC TATTAGCTAC TCTGTAACCC AGGGTGACCT TGAAGTCATG GGTAGCCTGC
GTTGATCTG ATAATCGATG AGACATTGGG TCCCACTGGA ACTTCAGTAC CCATCGGACG

3361 TGTTTTAGCC TTCCACATC TAAGATTACA GGTATGAGCT ATCATTTTTG GTATATTGAT
ACAAAATCGG AAGGGTGTAG ATTCTAATGT CCATACTCGA TAGTAAAAAC CATATAACTA

3421 TGATTGATTG ATTGATGTGT GTGTGTGTGA TTGTGTTTGT GTGTGTGANT GTGWANATGT
ACTAACTAAC TAACTACACA CACACACACT AACACAAACA CACACACTNA CACWTNTACA

3481 GTGTATGGNT GTGTGTGAKT GTGTGTATGT ATGNYTGTGT GTGANTGYGT GTGTGTGANT
CACATACCNA CACACACTMA CACACATACA TACNRACACA CACTNACRCA CACACACTNA

3541 GTGCATGTGT GTGTGTGTGA CTGTGTCTAT GTGTATGACT GTGTGTGTGT GTGTGTGTGT
CACGTACACA CACACACACT GACACAGATA CACATACTGA CACACACACA CACACACACA

3601 GTGTGTGTGT GTGTGTGTGT GTGTGTTGTG AAAAAATATT CTATGGTAGT GAGAGCCAAC
CACACACACA CACACACACA CACACAACAC TTTTTTATAA GATACCATCA CTCTCGGTTG

3661 GCTCCGGCTC AGGTGTCAGG TTGGTTTTTG AGACAGAGTC TTTCACCTAG CTGGAATTC
CGAGGCCGAG TCCACAGTCC AACCAAAAAC TCTGTCTCAG AAAGTGAATC GAACCTTAAG

3721 TTGAAGACGA AAGGGCCTCG TGATACGCCT ATTTTTATAG GTTAATGTCA TGATAATAAT
AACTTCTGCT TTCCGGGAGC ACTATGCGGA TAAAAATATC CAATTACAGT ACTATTATTA

3781 GGTTCCTTAG ACGTCAGGTG GCACTTTTTCG GGGAAATGTG CGCGGAACCC CTATTTGTTT
CCAAAGAATC TGCAGTCCAC CGTGAAAAGC CCCTTTACAC GCGCCTTGGG GATAAACAAA

3841 ATTTTCTAA ATACATTCAA ATATGTATCC GTCATGAGA CAATAACCCT GATAAATGCT
TAAAAGATT TATGTAAGTT TATACATAGG CGAGTACTCT GTTATTGGGA CTATTACGA

3901 TCAATAATAT TGA AAAAGGA AGAGTATGAG TATTCAACAT TTCCGTGTGCG CCCTTATTCC
AGTTATTATA ACTTTTTCCT TCTCATACTC ATAAGTTGTA AAGGCACAGC GGAATAAGG

3961 CTTTTTTGCG GCATTTTGCC TTCCTGTTTT TGCTACCCA GAAACGCTGG TGAAAGTAAA
GAAAAACGC CGTAAAACGG AAGGACAAAA ACGAGTGGGT CTTTGCGACC ACTTTCATTT

4021 AGATGCTGAA GATCAGTTGG GTGCACGAGT GGGTTACATC GAACTGGATC TCAACAGCGG
TCTACGACTT CTAGTCAACC CACGTGCTCA CCCAATGTAG CTTGACCTAG AGTTGTCCCG

FIG.17E

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4081 TAAGATCCTT GAGAGTTTTC GCCCCGAAGA ACGTTTTCCA ATGATGAGCA CTTTAAAGT
ATTCTAGGAA CTCTCAAAG CCGGGCTTCT TGCAAAGGT TACTACTCGT GAAAATTTCA

4141 TCTGCTATGT GCGCGGTAT TATCCCGTGT TGACCGCGG CAAGAGCAAC TCGGTGCGG
AGACGATACA CCGCGCCATA ATAGGCACA ACTGCGGCC GTTCTCGTTG AGCCAGCGG

4201 CATACTAT TCTCAGAATG ACTTGGTTGA GTACTACCA GTCACAGAAA AGCATCTTAC
GTATGTGATA AGAGTCTTAC TGAACCACT CATGAGTGGT CAGTGTCTTT TCGTAGAATG

4261 GGATGGCATG ACAGTAAGAG AATTATGCAG TGCTGCCATA ACCATGAGTG ATAACACTGC
CCTACCGTAC TGTATTCTC TTAATACGTC ACGACGGTAT TGGTACTCAC TATTGTGACG

4321 GGCCAACTTA CTTCTGACAA CGATCGGAGG ACCGAAGGAG CTAACCGCTT TTTTGACAA
CCGGTTGAAT GAAGACTGT TCTAGCCTCC TGGCTTCCTC GATTGGCGAA AAAACGTGT

4381 CATGGGGGAT CATGTAATC GCCTTGATCG TTGGGAACCG GAGCTGAATG AAGCCATACC
GTACCCCTA GTACATTGAG CGGAAGTAG AACCTTGGC CTCGACTTAC TTCGGTATGG

4441 AAACGACGAG CGTGACACCA CGATGCCTGC AGCAATGGCA ACAACGTTGC GCAAATATT
TTTGCTGCTC GCACTGTGCT GCTACGGAGC TCGTTACCGT TGTGCAACG CGTTTGATAA

4501 AACTGGCGAA CTAATTACTC TAGCTTCCCG GCAACAATTA ATAGACTGGA TGGAGGCGGA
TTGACCGCTT GATGAATGAG ATCGAAGGGC CGTTGTTAAT TATCTGACCT ACCTCGCCT

4561 TAAAGTTGCA GGACCACTTC TGGCTCGGC CCTTCCGGCT GGCTGGTTTA TTGCTGATAA
ATTTCAACGT CCTGGTGAAG ACGGAGCCG GGAAGGCCG CCGACCAAT AACGACTATT

4621 ATCTGGAGCC GGTGAGCGTG GGTCTCGCG TATCATTGCA GCACTGGGGC CAGATGGTAA
TAGACCTCGG CCACTCGCAC CCAGAGCGCC ATAGTAACGT CGTGACCCCG GTCTACCATT

4681 GCCCTCCCGT ATCGTAGTTA TCTACACGAC GGGGAGTCAG GCAACTATGG ATGAACGAAA
CGGGAGGGCA TAGCATCAAT AGATGTGCTG CCCCTCAGTC CGTTGATACC TACTTGCTTT

4741 TAGACAGATC GCTGAGATAG GTGCCTCACT GATTAAGCAT TGGTAACTGT CAGACCAAGT
ATCTGTCTAG CCACTCTATC CACGGAGTGA CTAATTCGTA ACCATTGACA GTCTGGTTCA

FIG.17F

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4801 TTAATCATAT ATACTTTAGA TTGATTTAAA ACTTCATTTT TAATTTAAAA GGATCTAGGT
AATGAGTATA TATGAAATCT AACTAAATTT AGAAGTAAAA ATTAAATTTT CCTAGATCCA

4861 GAAGATCCTT TTTGATAATC TCATGACCAA AATCCCTTAA CGTGAGTTTT CGTTCCACTG
CTTCTAGGAA AACTATTAG AGTACTGGTT TTAGGGAATT GCACTCAAAA GCAAGGTGAC

4921 AGCGTCAGAC CCCGTAGAAA AGATCAAAGG ATCTTCTTGA GATCCTTTTT TTCTGCGCGT
TCGCAGTCTG GGGCATCTTT TCTAGTTTCC TAGAAGAACT CTAGGAAAAA AAGACGCGCA

4981 AATCTGCTGC TTGCAAACAA AAAAACCACC GCTACCAGCG GTGGTTTGTG TGCCGGATCA
TTAGACGACG AACGTTTGTG TTTTGGTGG CGATGGTCCG CACCAACAA ACGGCCTAGT

5041 AGAGCTACCA ACTCTTTTTC CGAAGGTAAC TGGCTTCAGC AGAGCGCAGA TACCAAATAC
TCTCGATGGT TGAGAAAAAG GCTTCCATTG ACCGAAGTCC TCTCGCGTCT ATGGTTTATG

5101 TGTCTTCTA GTGTAGCCGT AGTTAGGCCA CCACTTCAAG AACTCTGTAG CACCGCCTAC
ACAGGAAGAT CACATCGGCA TCAATCCGGT GGTGAAGTTC TTGAGACATC GTGGCGGATG

5161 ATACCTCGCT CTGCTAATCC TGTTACCAGT GGCTGCTGCC AGTGGCGATA AGTCGTGTCT
TATGGAGCGA GACGATTAGG ACAATGGTCA CGACGACGG TCACCGCTAT TCAGCACAGA

5221 TACCGGGTTG GACTCAAGAC GATAGTTACC GGATAAGCGG CAGCGGTCCG GCTGAACGGG
ATGGCCCAAC CTGAGTTCTG CTATCAATGG CCTATTCCCG GTCGCCAGCC CGACTTGCCC

5281 GGGTTCTGTC ACACAGCCCA GCTTGGAGCG AACGACCTAC ACCGAACTGA GATACCTACA
CCCAAGCAGG TGTGTGGGT CGAACCTCGC TTGCTGGATG TGGCTTGACT CTATGGATGT

5341 GCGTGAGCTA TGAGAAAGCG CCACGCTTCC CGAAGGGAGA AAGCGGACA GGTATCCGGT
CGCACTCGAT ACTCTTTCGC GGTGCGAAGG GCTTCCCTCT TTCCGCCTGT CCATAGGCCA

5401 AAGCGGCAGG GTCGGAACAG GAGAGCGCAC GAGGGAGCTT CCAGGGGGAA ACGCCTGGTA
TTCCCGTCC CAGCCTTGTC CTCTCGGTG CTCCCTCGAA GGTCCCCCTT TCGGACCAT

5461 TCTTTATAGT CCTGTGGGT TTCGCCACCT CTGACTTGAG CGTCGATTTT TGTGATGCTC
AGAAATATCA GGACAGCCCA AAGCGGTGGA GACTGAACTC GCAGCTAAAA AACTACGAG

5521 GTCAGGGGGG CGGAGCCTAT GGAAAAACGC CAGCAACCGG GCCTTTTTAC GGTTCCTGGC
CAGTCCCCCC GCCTCGGATA CCTTTTTCG GTCGTTGCCG CGGAAAAATG CCAAGGACCG

FIG.17G

SUBSTITUTE SHEET (RULE 26)

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5581 CTTTTGCTGG CCTTTTGCTC ACATGTTCTT TCCTGCGTTA TCCCCTGATT CTGTGGATAA
GAAAACGACC GGAAAACGAG TGTACAAGAA AGGACGCAAT AGGGGACTAA GACACCTATT

5641 CCGTATTACC GCCTTTGAGT GAGCTGATAC CGCTCGCCGC AGCCGAACGA CCGAGCGCAG
GGCATAATGG CGGAAACTCA CTCGACTATG GCGAGCGGCG TCGGCTTGCT GGCTCGCGTC

5701 CGAGTCAGTG AGCGAGGAAG CGGAAGAGCG CCTGATGCGG TATTTTCTCC TTACGCATCT
GCTCAGTCAC TCGCTCCTTC GCCTTCTCGC GGACTACGCC ATAAAAGAGG AATGCGTAGA

5761 GTGCGGTATT TCACACCGCA TATGGTGCAC TCTCAGTACA ATCTGCTCTG ATGCCGCATA
CACGCCATAA AGTGTGGCGT ATACCACGTG AGAGTCATGT TAGACGAGAC TACGGCGTAT

5821 GTTAAGCCAG TATACACTCC GCTATCGCTA CGTGA CTGGG TCATGGCTGC GCCCGACAC
CAATTCGGTC ATATGTGAGG CGATAGCGAT GCACTGACCC AGTACCGACG CGGGGCTGTG

5881 CCGCCAACAC CCGCTGACGC GCCCTGACGG GCTTGTCTGC TCCCGGCATC CGCTTACAGA
GGCGGTTGTG GGCGACTGCG CGGGACTGCC CGAACAGACG AGGGCCGTAG GCGAATGTCT

5941 CAAGCTGTGA CCGTCTCCGG GAGCTGCATG TGTCAGAGGT TTTCACCGTC ATCACCGAAA
GTTTCGACACT GGCAGAGGCC CTCGACGTAC ACAGTCTCCA AAAGTGGCAG TAGTGGCTTT

6001 CGCGCGAGGC AGCTGCGGTA AAGCTCATCA GCGTGGTCTG GAAGCGATTG ACAGATGTCT
GCGCGCTCCG TCGACGCCAT TTCGAGTAGT CGCACCAGCA CTTCGCTAAG TGTCTACAGA

6061 GCCTGTTTAT CCGCGTCCAG CTCGTTGAGT TTCTCCAGAA GCGTTAATGT CTGGCTTCTG
CGGACAAGTA GGCGCAGGTC GAGCAACTCA AAGAGGTCTT CGCAATTACA GACCGAAGAC

6121 ATAAAGCGGG CCATGTTAAG GGCGGTTTTT TCCTGTTTGG TCACTTGATG CCTCCGTGTA
TATTTGCCCC GGTACAATTC CCGCCAAAAA AGGACAAACC AGTGAAC TAC GGAGGCACAT

6181 AGGGGGAATT TCTGTTTATG GGGGTAATGA TACCGATGAA ACGAGAGAGG ATGCTCACGA
TCCCCCTTAA AGACAAGTAC CCCCATTACT ATGGTACTT TGCTCTCTCC TACGAGTGCT

6241 TACGGGTTAC TGATGATGAA CATGCCCGGT TACTGGAACG TTGTGAGGGT AAACAAC TGG
ATGCCCAATG ACTACTACTT GTACGGGCCA ATGACCTTGC AACACTCCCA TTTGTTGACC

6301 CCGTATGGAT GCGGCGGGAC CAGAGAAAAA TCACTCAGGG TCAATGCCAG CGCTTCGTTA
GCCATACCTA CGCCGCCCTG GTCTCTTTTT AGTGAGTCCC AGTTACGGTC GCGAAGCAAT

FIG.17H

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6361 ATACAGATGT AGGTGTTCCA CAGGGTAGCC AGCAGCATCC TCGATGCAG ATCCGGAACA
TATGTCTACA TCCACAAGGT GTCCCATCGG TCGTCGTAGG ACGCTACGTC TAGGCCTTGT

6421 TAATGGTGCA GGGCGCTGAC TTCCGCGTTT CCAGACTTTA CGAAACACGG AAACCGAAGA
ATTACCAAGT CCCGCGACTG AAGGCGCAA GGTCTGAAAT GCTTTGTGCC TTTGGCTTCT

6481 CCATTATGT TGTGCTCAG GTCGCAGACG TTTTGCAGCA GCAGTOGCTT CACGTTGCT
GGTAAGTACA ACAACGAGTC CAGCGTCTGC AAAACGTCGT CGTCAGCGAA GTGCAAGCGA

6541 CGCGTATCGG TGATTCATTC TGCTAACCAG TAAGGCAACC CCGCCAGCCT AGCCGGGTCC
GCGCATAGCC ACTAAGTAAG ACGATTGGTC ATTCCGTTGG GCGGTCGGA TCGGCCCAGG

6501 TCAACGACAG GAGCAGATC ATGCGCACCC GTGGCCAGGA CCCAACGCTG CCCGAGATGC
AGTTGCTGTC CTCGTGCTAG TACCGTGGG CACCGTCCT GGGTTGCGAC GGGCTCTACG

6561 GCCGCGTGG GCTGCTGGAG ATGGCGGACG CGATGGATAT GTTCTGCCAA GGGTTGTTT
CGGCGCACGC CGACGACCTC TACCGCCTGC GCTACCTATA CAAGACGGTT CCCAACCAAA

6721 GCGCATTAC AGTTCTCCG AAGAATTGAT TGGCTCCAAT TCTTGAGTG GTGAATCCGT
CGCGTAAGTG TCAAGAGGCG TTCTTAATA ACCGAGGTA AGAACCTCAC CACTTAGGCA

6781 TAGCGAGGTG CCGCCGGCTT CCATTACAGT CGAGGTGGCC CGGCTCCATG CACCGCGACG
ATCGTCCAC GCGGCGCGAA GGTAAAGTCA GCTCCACCG GCGAGGTAC GTGGCGCTGC

6841 CAACGCGGG AGGCAGACAA GGTATAGGGC GGCGCTACA ATCCATGCCA ACCCGTTCCA
GTTGCGCCCC TCCGTCTGTT CCATATCCCG CCGCGGATGT TAGGTACGGT TGGCAAGGT

6901 TGTGCTCGCC GAGGCGGCAT AAATCGCGT GAGATCAGC GGTCCAGTGA TCGAAGTTAG
ACACGAGCGG CTCGCGCGTA TTAGCGGCA CTGCTAGTCG CCAGGTCCT AGCTTCAATC

6961 GCTGGTAAGA GCGCGAGCG ATCCTTGAAG CTGTCCCTGA TGGTCGTCAT CTACCTGCCT
CGACCATCT CCGCGCTCG TAGGAAGTTC GACAGGGACT ACCAGCAGTA GATGGACGGC

7021 GGACAGCATG GCCTGCAACG CGGGCATCCC GATGCCGCG GAAGCGAGAA GAATCATAAT
CCTGTCGTAC CGGACGTTGC GCCCGTAGGG CTACGGCGC CTTGCTCTT CTTAGTATTA

7081 GGGGAAGGCC ATCCAGCCTC GCGTCGCGAA CGCCAGCAAG ACGTAGCCCA GCGCGTCGGC
CCCCTTCCG TAGGTGCGAG CGCAGCGCTT GCGGTCGTT TGCATCGGT CCGCAGCCG

7141 CGCCATGCCG GCGATAATGG CCTGCTTCTC GCCGAAACGT TTGGTGGCG GACCAGTGAC
CGGTACGGC CGCTATTACC GGACGAAGAG CGGCTTTGCA AACCACCGC CTGGTCACTG

FIG.171

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7201 GAAGGCTTGA GCGAGGGCGT GCAAGATTCC GAATACCGCA AGCGACAGGC CGATCATOGT
CTTCCGAAC TCGTCCCGCA CGTTCTAAGG CTTATGGCGT TCGCTGTCCG GCTAGTAGCA

7261 CGCGCTCCAG CGAAAGCGGT CCTCGCCGAA AATGACCCAG AGCGCTGCCG GCACCTGTCC
GCGGAGGTC GCTTTCGCCA GGAGGGGCTT TTAGTGGGTC TCGCGACGGC CGTGGACAGG

7321 TACGAGTTGC ATGATAAAGA AGACAGTCAT AAGTGCGGCG ACGATAGTCA TGCCCCGCGC
ATGCTCAACG TACTATTTCT TCTGTCAGTA TTCACGCGCG TGCTATCAGT ACGGGGCGCG

7381 CCACCGGAAG GAGCTGACTG GGTGAAGGC TCTCAAGGGC ATCGGTGAC GCTCTCCCTT
GGTGGCCTTC CTCGACTGAC CCAACTTCCG AGAGTTCCCG TAGCCAGCTG CGAGAGGGAA

7441 ATCGGACTCC TGCATTAGGA AGCAGCCCAG TAGTAGGTTG AGGCCGTTGA GCACCGCCGC
TAGCTGAGG ACGTAATCCT TCGTCGGGTC ATCATCCAAC TCCGGCAACT CGTGGCGGCG

7501 CGCAAGGAAT GGTGCATGCA AGGAGATGGC GCCCAACAGT CCCCCGGCCA CGGGGCCTGC
GCGTTCCTTA CCACGTACGT TCCTCTACCG CGGGTTGTCA GGGGGCCGGT GCCCGGACG

7561 CACCATACCC ACGCCGAAAC AAGCGCTCAT GAGCCCGAAG TGGCGAGCCC GATCTTCCCC
GTGGTATGGG TCGGCTTTG TCGCGAGTA CTCGGGCTTC ACCGCTCGGG CTAGAAGGGG

7621 ATCGGTGATG TCGGCGATAT AGGCGCCAGC AACCGCACCT GTGGCGCCGG TGATGCCGGC
TAGCCACTAC AGCCGCTATA TCCGCGGTCC TTGGCGTGA CACCGCGGCC ACTACGGCCG

7681 CACGATGCGT CCGGCGTAGA GCGCCACAGG ACGGGTGTGG TCGCCATGAT CGCGTAGTCG
GTGCTACGCA GGCCGCATCT CGCGGTGTCC TGCCACACC AGCGGTACTA GCGCATCAGC

7741 ATAGTGGCTC CAAGTAGCGA AGCGAGCAGG ACTGGGCGGC GGCCAAAGCG GTCGGACAGT
TATCACCGAG GTTCATCGCT TCGCTCGTCC TGACCCGCGC CCGGTTTCG CAGCCTGTCA

7801 GCTCCGAGAA CGGGTGCGCA TAGAAATTGC ATCAACGCAT ATAGCGCTAG CAGCAGCCA
CGAGGCTCTT GCCACGCGT ATCTTTAACG TAGTTGCGTA TATCGCGATC GTCGTGCGGT

7861 TAGTGA CTGCTGTC GGAATGGACG ATATCCCGCA AGAGGCCCGG CAGTACCGGC
ATCACTGACC GCTACGACAG CCTTACCTGC TATAGGGCGT TCTCCGGGCC GTCATGGCCG

7921 ATAACCAAGC CTATGCCTAC AGCATCCAGG GTGACGGTGC CGAGGATGAC GATGAGCGCA
TATTGGTTCC GATACGGATG TCGTAGGTCC CACTGCCACG GCTCCTACTG CTA CTGCGT

7981 TTGTTAGATT TCATACACCG TGCCTGACTG CGTTAGCAAT TTAAGTGTGA TAACTACCG
AACAACTAA AGTATGTGCC ACGGACTGAC GCAATCGTTA AATTGACACT ATTTGATGGC

7041 CATTA
GTAAT

FIG.17J
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